EVOLUTION OF HORMONE-RECEPTOR SYSTEMS

Ralph A. Bradshaw and Gordon N. Gill, Organizers

March 14 – March 20, 1982

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Cell Surface Receptors Regulating Cell Growth

0303 STRUCTURE AND FUNCTION OF THE NERVE GROWTH FACTOR RECEPTOR OF PERIPHERAL NEURONS. Ralph A. Bradshaw, Department of Biological Chemistry, Washington University School of Medicine, St. Louis MO 63110

Nerve growth factor (NGF) is one of many polypeptide agents that interact with a spectrum of target cells to modulate activities related to or required for the growth response, i.e. hypertrophy or hyperplasia. The initiation of the molecular events that comprise these activities appears to universally require the specific binding to a membrane-bound recognitive entity which in turn transmits the signal(s) to the interior of the cell. Although the characterization of these receptor molecules is still in the early stages, it is already clear that there is more diversity than similarity in their gross structural features. The NGF receptor from peripheral neurons also follows this pattern. As shown by both hydrodynamic and affinity cross-linking studies, it has a molecular weight of ∿130,000. The latter experiments also revealed a lower molecular weight species (∿100,000 Mr), present in variable amounts, that appears to be a limited proteolytic product of the 130K species. Whether it might represent a physiological significant form of the receptor remains to be determined. These properties, when contrasted with those of the receptors of other growth factors, are, at present, unique. Even the hormones related to NGF through a common ancestor, i.e. insulin and insulin-like growth factors I and II, have receptors apparently quite dissimilar to NGF. The receptor for a fifth member of this evolutionarily-related subset, relaxin, remains uncharacterized. These findings suggest that the development of hormones and growth factors via gene duplication, splicing and mutation may not have been paralleled by similar events in the development of their receptors. Such independent lines of evolution may have introduced important elements of diversity in the molecular aspects of growth regulation. Supported by U.S.P.H.S. research grant NS-10229.

0304 REGULATION OF MONONUCLEAR PHAGOCYTE GROWTH BY A COLONY STIMULATING FACTOR (CSF-1) E.R. Stanley, S.H. Bartelmez, P.V. Byrne, L.J. Guilbert, I.T. Oliver, R.J. Tushinski and P.W. Tynan, Departments of Microbiology & Immunology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461.

Mature mononuclear phagocytes (blood monocytes and tissue macrophages) are derived from the hemopoietic stem cell by a process of proliferation and differentiation (stem cell→→→precursor cell→monoblast→promonocyte→monocyte→macrophage). Each stem cell can give rise to many macrophages and the role of growth factors in this process is under investigation in this laboratory.

Proliferation of the more differentiated cells (monoblast→macrophage) is regulated by a circulating glycoprotein growth factor (CSF-1) (1). CSF-1 (M_r 45,000-70,000) consists of 2 similar subunits that are linked by disulfide bonds. N-glycosidically linked 'acidic complex' carbohydrate moieties contribute as much as 50% to the M_r of the molecule. Specific cell surface receptors for CSF-1 occur only on mononuclear phagocytic cells and mediate the biological effects of the growth factor (2). Past studies on the mechanism of CSF-1 action have been limited because the proliferative responses of individual freshly explanted macrophages vary. Homogeneous macrophage populations were obtained by culturing bone marrow cells in the presence of CSF-1 preparations and selecting for cells that had recently acquired the ability to adhere to a substratum (3). These bone marrow derived macrophages (BMM) require CSF-1 for both their survival and grwoth in serum-containing cultures. The presence of CSF-1 during G_1 is both necessary and sufficient for the entry of BMM into S-phase and their progression through G_2 and M (4). Compared with non-proliferating BMM, BMM proliferating in the presence of CSF-1 are extensively vacuolated. In addition, they rapidly and continuously destroy CSF-1 via the CSF-1 receptor. Thus survival, growth and differentiation of mononuclear phagocytes is dependent on a lineage specific growth factor which the differentiated cells selectively destroy.

Three factors have been resolved which increase the numbers of more primitive CSF-1 binding cells (precursor cells→monoblasts) by stimulating proliferation of as yet unidentified precursor cells in bone marrow cell cultures. They are CSF-1 itself, a factor which acts independently of CSF-1 and another factor, the action of which requires the presence of CSF-1. Rapid assays for each of these factors involve measurement of the increase in total ^{125}I-CSF-1 binding by bone marrow cells following a 3 day incubation.

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0305 ENERGY DEPENDENT GENERATION OF A HIGH AFFINITY SUBCLASS OF EGF RECEPTORS IN ISOLATED MEMBRANE VESICLES, C. Fred Fox, Ken Iwata, Michael Lipari, Hilda Chamras and Shintaro Iwashita, Molecular Biology Institute and Department of Microbiology, University of California, Los Angeles, CA 90024
Scatchard analysis of EGF bound to murine 3T3 cells reveals two classes of specifically associated hormone: approximately 80% in a low affinity class (K_d = 10nM) and 20% in a high affinity class (K_d = 0.1nM). Binding to the high affinity class correlates with the 50% effective dose for EGF induced mitogenesis, also 0.1nM. The high affinity class is not detected on 3T3 cells incubated with EGF for short times or at low temperature, but is when cells given prior treatment with unlabeled EGF are then incubated with labeled EGF. Energy poisons and phorbol esters block high affinity binding without decreasing total binding, but appearance of the high affinity class is not affected by protein synthesis inhibitors. These studies show that the high affinity binding class of EGF receptors is formed in cells in a process requiring energy, but not protein synthesis.

High affinity (0.1nM K_d) EGF receptors are not present on membranes derived from 3T3 cells grown with calf serum or in serum free medium. Addition of unlabeled EGF during the final stages of growth induces high affinity EGF receptors detected in subsequent binding experiments with membranes isolated from EGF-treated cells. Since energy poisons block formation of the high affinity class of EGF binding on cells, and since EGF receptors are phosphorylated by ATP, we tested for ATP-dependent formation of high affinity EGF receptors in membranes isolated from cells displaying low affinity EGF receptors only. High affinity EGF receptors are formed in vitro in an ATP-dependent reaction induced by EGF.

More recently we have studied phosphorylation of EGF receptors in isolated membranes in attempts to relate receptor phosphorylation to formation of the high affinity receptor subclass. Two dimensional fingerprinting of tryptic digests of the high molecular weight EGF receptor band reveals 11 major phosphopeptides; most of these contain one or the other of P-tyr, P-ser or P-thr. EGF stimulates the phosphorylation of all these, but phosphorylation of some species is more highly influenced by EGF than is that of others. Studies in progress are testing the possible relationship of enhanced phosphorylation in specific receptor P-peptides with formation of a high affinity subclass of EGF receptors. (Supported by grants from USPHS, ACS and MDAA).

0306 HOMOLOGOUS SUBUNIT STRUCTURES OF THE RECEPTORS FOR INSULIN AND INSULIN-LIKE GROWTH FACTOR I SUGGEST A COMMON EVOLUTIONARY ORIGIN, Michael P. Czech, Joan Massague, Julia Heinrich, and Laura A. Blinderman, Department of Biochemistry, University of Massachusetts Medical Center, Worcester, Massachusetts 01605
Affinity crosslinking methods developed in our laboratory have proven useful in specifically labeling several cell surface receptors and transport systems. Our studies on the subunit structures of the affinity-labeled receptors for insulin and IGF-I has led to the conclusion that these respective receptors share a number of striking structural similarities in spite of their divergent affinities for hormones. The insulin receptor exhibits a high affinity for insulin and a low affinity for IGF-I or IGF-II while the IGF-I receptor has a high affinity for IGF-I, a lower affinity for IGF-II, and a still lower affinity for insulin. The two receptors share the following structural features: 1. The minimum receptor subunit structures consist of two subunit types denoted as α (Mr=125,000) and β (Mr=90,000) in a disulfide-linked heterotetrameric structure (β-S-S-α)-S-S-(α-S-S-β), 2. Indirect evidence indicates both α and β subunits in these receptors are glycoproteins, 3. The center disulfide linkages (class I) that connect the two (β-S-S-α) receptor halves are sensitive to exogenous reductants in native cells or plasma membranes while the disulfides (class II) linking the α and β subunits are not, 4. The class I disulfides are more sensitive to low concentrations of dithiothreitol than the class II disulfides when the receptors are solubilized in dodecyl sulfate, 5. The β subunits of both receptors contain a site near the center of their polypeptide chains that is extremely sensitive to proteolytic cleavage by elastase-like proteases. Cell screening studies indicate chick embryo fibroblasts and strain E Ascites carcinoma cells contain the IGF-I receptor but no insulin receptor. Rat adipocytes, rat hepatocytes, and cultured H35 hepatoma cells contain insulin receptor but no IGF-I receptor. A large number of cell types contain both receptor types. Two dimensional gel electrophoresis of membranes from cells that contain only one of the receptor types allows large scale purification of the receptors for further structural analysis. Results involving careful dose response relationship determinations for [^{3}H]-thymidine incorporation into DNA and cell proliferation demonstrate that the IGF-I receptor and the insulin receptor can mediate growth effects in the chick embryo fibroblast and H35 hepatoma cell, respectively. We conclude that marked structural similarities are characteristic of the insulin and IGF-I receptors and that this may reflect utilization of common gene sequences or the occurrence of gene duplication during evolution. The homologous receptor structures for these ligands and the ability of both receptors to mediate at least one similar biological effect (cell growth) in certain cell types also implies the utilization of similar mechanisms of transmembrane signalling.

Receptor Endocytosis, Metabolism, and Turnover

0307 THE LOW DENSITY LIPOPROTEIN RECEPTOR - STRUCTURE-FUNCTION RELATIONSHIPS, Wolfgang J. Schneider, Ulrike Beisiegel, Joseph L. Goldstein, and Michael S. Brown, Department of Molecular Genetics, University of Texas Health Science Center, Dallas, TX 75235

Normal human fibroblasts and many other mammalian cells express on their surface a receptor that binds low density lipoprotein (LDL). Subsequent uptake and lysosomal degradation of LDL are achieved by receptor-mediated endocytosis (1). In our effort to delineate the biochemical mechanisms of this process, we have purified the LDL receptor from bovine adrenal cortex (2), and we have begun its structural characterization. The receptor is a glycoprotein with an isoelectric point of pH 4.6. The single polypeptide chain has an apparent MW of 164,000. Upon treatment with neuraminidase, the electrophoretic mobility of the protein on SDS-polyacrylamide gels is significantly increased. Desialylation does not affect the ability of the receptor to bind LDL. Also, we have reconstituted the purified LDL receptor from bovine adrenal into phospholipid vesicles, and we have initiated studies on the orientation of the polypeptide in reconstitutes. An important tool in these studies is the use of monoclonal antibodies as probes for different regions of the receptor. We have prepared several monoclonal antibodies that recognize a common determinant on the human fibroblast and bovine adrenal receptor (3). One antibody, designated C7, was studied extensively. We have determined that on human fibroblasts and on the bovine receptor there is one antibody binding site per binding site for LDL. Furthermore, in human fibroblasts the monoclonal antibody is bound, internalized, and degraded in complete analogy to the receptor-mediated endocytosis of LDL. Interestingly, C7 and LDL apparently do not bind to identical sites on the receptor, as indicated by the following observations. When the receptor was fully occupied by prior incubation with LDL, ^{125}I-C7 was still able to bind in normal amounts. Moreover, when ^{125}I-LDL was pre-bound to the receptor at 4°C, the addition of a saturating amount of C7 did not cause the LDL to dissociate. In contrast, when the cells were first allowed to bind C7 at 4°C, the subsequent binding of ^{125}I-LDL was reduced by about 80%. Similar studies were performed with the reconstituted bovine LDL receptor. In addition to using C7 as a probe for functional sites on the receptor, it helped us to identify the human fibroblast LDL receptor. By blotting analysis, the fibroblast receptor appears to have the same electrophoretic mobility as the bovine adrenal receptor. Thus, LDL receptors from different species possess common features that can be identified immunologically. Monoclonal antibodies may serve as probes for common functional and structural domains of LDL receptors from a variety of sources.

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0308 RECEPTOR REDISTRIBUTION, INTERNALIZATION AND PROCESSING IN THE ACTION OF EGF, Pedro Cuatrecasas, Molecular Biology Department, The Wellcome Research Laboratories, Research Triangle Park, NC 27709

Using fluorescently labeled analogs of EGF, lateral diffusion of receptors along the plane of the membrane can be measured using the fluorescence photobleaching recovery method in cultured cells. With the aid of high image-intensified fluorescent microscopy, the receptors, which are initially distributed in a diffuse fashion, form patches and undergo endocytosis at 37°. These processes are probably not critical in mediating the initial, rapid actions of the hormone. By biochemical methods, the processes of uptake and endocytosis correspond to the loss of surface receptors ("down-regulation") and degradation of the receptor and hormone.[1] The hormone appears to be internalized via coated pits, although the intracellular vesicles ("receptosomes") are not coated with clathrin.[2] These vesicles migrate toward the Golgi-GERL and slowly transfer their content into small, new lysosomes. The EGF receptors are not apparently re-cycled or re-utilized, and they are continuously internalized, even in the absence of ligand.[1] Transglutaminase does not appear to be necessary for endocytosis. Since all maneuvers that interfere with intracellular degradation and processing block mitogenesis, it is proposed that these may be essential processes, although in such a case they must be continuous and protracted functions.[3] Slow nuclear accumulation of the H-R complex may be an important process.[4] In addition, evidence exists to suggest that limited (submicroscopic) receptor aggregation (dimerization) at the cell surface may be sufficient to trigger the immediate biological effects and may also be required for endocytosis.[5] The ligand itself may not be an essential structural component of the action of the receptor. Recent data suggest that the accumulation of a stable intracellular complex may be directed by a special population of very high affinity receptors that control cellular growth and tumor promotion.[6]

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0309 PROTEIN-PROTEIN INTERACTIONS IN RECEPTOR-MEDIATED ENDOCYTOSIS, Thomas F. Roth, John W. Woods, and John Daiss, Department of Biological Sciences, University of Maryland Baltimore County (UMBC), Catonsville, MD 21228

In the 20 years since coated pits and vesicles were first discovered and postulated to be involved in selective protein transport, the field of receptor-mediated endocytosis (RME) has blossomed. Numerous examples have been described where coated pits and vesicles mediated the transport of polypeptides into cells. Further, virtually every cell type in higher animals exhibits some degree of RME which indicates that this process is an ancient and ubiquitous process. Several basic steps are involved. Ligand binding to specific cell-surface receptors, clustering of the receptors in coated pits, assembly of the coat at discrete regions of the membrane, invagination of the coated pit to form a coated vesicle, disassembly of the coat, intracellular sorting of the ligand to specific destinations and recycling of membrane receptors are common steps in many examples of RME.

We are pursuing the problems of receptor-ligand, receptor-coat and coat-coat interactions. Among the model systems we employ are the chicken oocyte and yolk sac in which we have operationally defined receptors for LDL, VLDL, vitellogenin and IgG. We are currently examining the localization of these receptors using electron dense labels. The receptor for vitellogenin has been tentatively identified and partially purified. At present, we are preparing anti-receptor antibodies to follow receptor interiorization, recycling and synthesis.

Coated vesicle components and the interactions among them are being investigated with zero-angstrom cross-linking agents and polypeptide-specific monoclonal antibodies. A model of the coated vesicle is presented illustrating our current understanding of coat protein interactions.

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Membrane-Receptor-Linked Systems

0310 STRUCTURAL ORGANIZATION OF THE CALMODULIN GENE, Anthony R. Means, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, Lisette Lagace, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, Joseph P. Stein, Division of Endocrinology, University of Texas Health Science Center, Houston, TX 77030.

The amino acid sequence of calmodulin (CaM), the ubiquitous intracellular Ca^{++} receptor, is highly conserved among representative species of both animal and plant kingdoms. Based on a significant degree of internal homology and the fact that each protein molecule binds 4 Ca^{++}, CaM has been proposed to contain 4 functional domains. These data have been used to develop a theory that the current CaM molecule has arisen by duplications of a primordial gene. We have cloned the chicken CaM gene in order to determine if this theory is supported by the position of intervening sequences within the gene since such introns have been reported to separate functional domains. A full-length cDNA was isolated, cloned and sequenced from mRNA derived from the electroplax tissue of the electric eel. This cDNA probe was used to screen a chicken DNA library for the presence of CaM genomic fragments. A 10.6 Kb fragment from one of the 8 positive clones was subcloned in pBR322 (pCM 1.3) and sequenced. The genomic fragment begins very near the 5' end of the calmodulin gene and extends into the 3' flanking region. 79% of the nucleotides prior to the translation termination signal are identical to the corresponding nucleotide of the eel calmodulin cDNA. When translated, 88% of the amino acid residues are identical in the two calmodulins. The DNA sequence confirms that no intervening sequences are present in the chicken calmodulin gene. Since internal sequence homologies argue that the present calmodulin gene has evolved by two gene duplication events. three alternatives can be envisioned to allow such an evolutionary change. First, the recombinational events could have occured within short "spacer regions", which are sequences between the domains that code for three or four amino acids that have not been assigned to the domains by amino acid homology. Although these amino acids might not be functionally critical, splicing would have to occur in such a manner so as to maintain the correct mRNA reading frame. Second, recombinational events occured with absolute precision in breakage of DNA immediately preceding and following the exon segments involved, in order to maintain the correct reading frame of the resulting mRNA. Since the recombination process itself is random, this would presumably be an event of low probability of occurrence. Third, recombination occured at any one of numerous sites in the flanking DNA on either side of the exon sequences. This would have led to the introduction of introns between the calmodulin domains, which then must have been lost since the formation of the primordial gene. It is clearly impossible at this time to distinguish experimentally between these three possibilities. Such evidence can only be obtained by sequencing calmodulin genes from diverse species.

0311 PHOSPHOLIPID TURNOVER AND CYCLIC NUCLEOTIDES: A RECEPTOR-LINKED CASCADE FOR BI-DIRECTIONAL CONTROL OF PROTEIN PHOSPHORYLATION, Yasutomi Nishizuka, Department of Biochemistry, Kobe University School of Medicine, Kobe 650, and Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan

There appear to be two major receptor functions which are responsible for activation and inhibition of specific functions and proliferation of a wide variety of mammalian cells. In such bidirectional control systems various cellular activities may be regulated by two opposing extracellular messengers. It has been recently clarified in this laboratory that phosphatidylinositol (PI) turnover, which is provoked by a group of extracellular messengers such as α-adrenergic and muscarinic cholinergic stimulators, peptide hormones, and many other biologically active substances including mitogens and growth factors, serves as a signal for the control of cellular processes through selective activation of a new species of protein kinase (C-kinase). The activation of this kinase absolutely requires Ca^{2+} and phosphatidylserine (PS). Under physiological conditions unsaturated diacylglycerol (DG) derived from the receptor-linked breakdown of PI dramatically increases the affinity of enzyme for Ca^{2+} as well as for PS, and thereby initiates C-kinase activation. Other phospholipids show positive or negative cooperativity in this process. C-Kinase activated in this way shows catalytic activities entirely different from those of cyclic AMP-dependent and cyclic GMP-dependent protein kinases (A- and G-kinases, respectively), but appears to show pleiotropic functions and phosphorylates many proteins such as those associated with membranes. A series of studies *in vitro* as well as *in vivo* with human platelets as a model system indicates that the thrombin-induced DG formation, C-kinase-specific phosphorylation and serotonin release reaction proceed in parallel manners, and evidence is now available indicating that such transmembrane control of protein phosphorylation does operate in cellular activation processes. In most cases, a part of DG (or phosphatidic acid therefrom) releases arachidonic acid to produce prostaglandin derivatives, which eventually modulate cyclic nucleotide levels. In fact, cyclic GMP has been shown to be often increased by this group of extracellular messengers, although the causal relationship between PI turnover and Ca^{2+} gate opening is not known at present. In contrast, cyclic AMP is normally increased by stimulation of the other receptor function and appears to act as an inhibitor for cellular activation and proliferation. It is also shown in the present studies that both cyclic AMP and cyclic GMP block the receptor-linked breakdown of PI to produce DG presumably through the action of A- and G-kinases, and thereby counteract the activation of C-kinase. Thus, it is attractive to propose that both cyclic AMP and cyclic GMP serve as negative signals which are involved in the extracellular and intracellular circuits, respectively, leading to the feedback control of cellular activation processes in such bidirectional control systems. (supported by the Ministry of Education, Science, and Culture, Japan)

Growth Factors and Cell Division

0312 TOXIC LIGAND CONJUGATES AS TOOLS IN THE STUDY OF RECEPTOR-LIGAND INTERACTIONS, Harvey Herschman, Daniel Cawley, and David Simpson, Department of Biological Chemistry, and Laboratory of Biomedical and Environmental Sciences, University of California, Los Angeles, Los Angeles, CA 90024.

Ricin, a toxin found in the castor bean, and diphtheria toxin have similar polypeptide structures. Each is composed of a B-chain which recognizes a cell-surface component and an A-chain which is able to catalytically inactivate a component of the protein-synthesizing apparatus of mammalian cells. The A and B chains of both ricin and diphtheria toxin are held together by a single disulfide bond. Although both ricin and diphtheria toxin are potent toxins on intact cells, neither the isolated B-chains nor A-chains of these molecules are effective toxins. We, and others, have attempted to make receptor-specific toxins by replacing the B-chains of ricin or diphtheria toxin with receptor-specific ligands such as hormones, growth factors, or antibodies.

In this presentation we will describe the synthesis of disulfide-linked conjugates of epidermal growth factor with the A-chains of ricin and diphtheria toxin. The EGF-ricin A-chain conjugate (EGF-RTA) is a potent toxin on cells bearing EGF receptors. In contrast the EGF-diphtheria toxin A-chain conjugate (EGF-DTA) is far less potent on 3T3 cells and A431 cells.

We have also prepared disulfide-linked conjugates of the two toxin A-chains with desialylated fetuin. Desialylated or asialoglycoproteins are recognized by a receptor found only on hepatocytes. Both the asialofetuin-ricin A-chain conjugate (ASF-RTA) and asialofetuin-diphtheria toxin A-chain (ASF-DTA) conjugates are (1) toxic to hepatocytes, (2) unable to kill cells lacking receptors for asialoglycoproteins, and (3) inhibited by desialylated glycoproteins but not by their native counterparts. These data demonstrate that the toxicity of ASF-RTA and ASF-DTA is mediated by the asialoglycoprotein receptor.

Because of the equivalent toxicity of the DTA and RTA conjugates of asialofetuin on hepatocytes, which contrasted with our previous observations on the paired EGF conjugates (EGF-RTA and EGF-DTA) on 3T3 cells and A431 cells, we tested the latter two conjugates on hepatocytes. In contrast to the results obtained with other cell types, EGF-RTA and EGF-DTA were both potent toxins on hepatocytes.

Our results will be discussed in the context of current concepts of receptor-mediated endocytosis and the mechanisms of productive toxin internalization. Potential uses of hybrid toxins in biology and medicine will be presented.

0313 STRUCTURAL HOMOLOGY BETWEEN MOUSE, RAT AND HUMAN TRANSFORMING GROWTH FACTORS, George J. Todaro, Hans Marquardt, Joseph E. De Larco and Daniel R. Twardzik, Laboratory of Viral Carcinogenesis, NCI, Frederick, MD 21701

Transforming growth factors (TGFs) that alter the properties of fibroblasts and epithelial cells and that interact with and induce the phosphorylation of EGF receptors have been isolated from the supernatant fluids of human tumor cells growing in culture. They have been purified by reverse phase high performance liquid chromatography using a C_{18}μBondapak column. TGFs have been isolated from the conditioned medium of a Moloney sarcoma virus-transformed mouse 3T3 cell line, an Abelson leukemia virus-transformed rat Fisher cell line, and a human melanoma cell line. Purification was achieved by gel permeation chromatography on Bio-Gel in 1 M acetic acid, followed by reverse phase high pressure liquid chromatography on μBondapak C_{18} support using aqueous acetonitrile and/or 1-propanol-trifluoroacetic acid as solvent systems. This has resulted in over a 200,000 fold purification.

Mouse, rat and human TGF are single chain polypeptides with an apparent molecular weight of 6,800. Partial sequence data is available using the gas phase sequenator developed at California Institute of Technology (M. Hunkapiller and L. Hood). From the data available the TGFs are clearly different from either mouse or human EGF. The sarcoma growth factor (SGF) isolated from Moloney sarcoma virus (MSV)-transformed mouse cells shows some sequence homology with human TGF and is also different from the EGFs.

TGFs are also detected in the urine of human cancer patients. These have been also partially purified and they, too, interact with the EGF receptor. We conclude that the transforming growth factors represent a family of biologically active peptides that are more potent growth stimulators than EGF and are distant relatives of EGF. The family of EGF-like compounds may prove to be as extensive as the family of polypeptides related to insulin and the insulin-like growth factors.

Regulation, Structure and Expression of Inducible Genes

0314 REGULATION OF GROWTH HORMONE AND PROLACTIN GENE EXPRESSION BY STEROIDS AND PEPTIDE HORMONES. Ronald M. Evans,* Marcia Barinaga,* Ellen Potter,* Estelita S. Ong,* Scott Supowit,§ Geoffrey Murdoch§ and Michael G. Rosenfeld.§ *The Salk Institute for Biological Studies, Tumor Virology Laboratory, San Diego, California 92138. §University of California, San Diego, Division of Endocrinology, School of Medicine, La Jolla, California 92093.

The production of growth hormone and prolactin is subject to complex control by both steroid and peptide hormones. The mechanisms and sites for this control have been examined using cDNA clones as hybridization probes to RNA isolated from cells following hormonal treatment. The rat growth hormone gene is shown to be regulated at a nuclear level by glucocorticoids while the prolactin gene is similarly controlled by the hypothalamic releasing factor TRH. We have explored the use of DNA mediated gene transfer to examine the nature of hormonal control. A novel retroviral cloning vehicle has been used to transfer the molecular clone of the growth hormone gene into mouse cells. The production of growth hormone by these cells is regulated by glucocorticoids and suggests that hormonal responsiveness is intrinsic to the structure of the gene or its RNA products. The use of these experiments to characterize hormonally inducible control elements will be discussed.

0315 HORMONAL CONTROL OF RAT α_{2u} GLOBULIN SYNTHESIS, David T. Kurtz and Douglas K. Bishop, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

α_{2u} Globulin is a male rat liver protein whose synthesis is under complex hormonal control in vivo. The protein is encoded by a large multigene family (18-20 genes per haploid complement) clustered on chromosome 5 (1).

Several α_{2u} globulin genes have been isolated from a library of the rat genome cloned in Charon 4A. The genes have been introduced into mouse Ltk$^-$ tissue culture cells and have been found to respond to dexamethasone (2). More recently, we have found that the transfected α_{2u} globulin genes also respond to insulin. Though insulin has no effect by itself, its inclusion along with dexamethasone results in a 5-10 fold greater induction of α_{2u} globulin mRNA in L cells than found with dexamethasone alone. This closely mimics the situation in a4/9 cells, a rat hepatoma line that synthesizes α_{2u} globulin (3).

Specific fragments from the 5' end of the α_{2u} globulin genes have been ligated to the structural gene for HSV thymidine kinase (TK). Preliminary results indicate that TK activity in cells containing these constructs is hormonally regulated.

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0316 ALTERNATIVE RNA PROCESSING EVENTS IN CALCITONIN GENE EXPRESSION, Michael G. Rosenfeld*, Susan G. Amara*, Vivian Jonas*, and Ronald M. Evans†, *Department of Medicine, University of California, San Diego, La Jolla, CA 92093; †The Salk Institute, San Diego, CA 92138

The neuroendocrine gene for calcitonin encodes an mRNA precursor which is alternatively spliced in a tissue-specific fashion to generate multiple mRNAs. The sequence of cDNA clones of two of the resultant mRNAs and the map and partial sequence of a genomic clone of the calcitonin gene are consistent with a model in which functional domains encoding component polypeptides are alternatively spliced during processing of the primary RNA transcript. Two mature mRNA species analyzed share an identical 5' coding domain but differentially contain one of two alternative 3' coding exons. As a result these mRNAs encode protein products having identical N-termini, but which can generate different peptides from their C-termini during proteolytic processing. The physiological and functional significance of these "peptide switching" events is suggested by the observation that the mRNA encoding the protein precursor of calcitonin represents the major species in C-cells of the thyroid, while a second alternatively spliced mRNA appears to be preferentially produced in the hypothalamus. The consequence of "peptide switching" is that one neuroendocrine gene can be expressed to generate the hormone calcitonin in the thyroid; and a new putative hormone, referred to as CGRP, in the hypothalamus.

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Hormone Receptors Which Act in the Nucleus

0317 RECEPTORS FOR THE VITAMIN D HORMONES-1,25-DIHYDROXYVITAMIN D_3 AND 24,25-DIHYDROXY-VITAMIN D_3. Anthony W. Norman, Department of Biochemistry, University of California, Riverside, California 92521

Vitamin D is a secosteroid which is a precursor to an endocrine system similar to those of classical steroid hormones. The active forms, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$], are formed from 25-hydroxyvitamin D_3 in the kidney via a tightly regulated mitochondrial 25(OH)-vitamin D-1-hydroxylase. In its target tissues, $1,25(OH)_2D_3$ interacts with an intracellular receptor protein which mediates genomic effects resulting in biological responses. This paper will principally report the results of several studies of the biochemical properties of receptors for $1,25(OH)_2D_3$. (a) A detailed assessment has been carried out of the structure-function requirements of receptors in the chick intestinal mucosa for over 36 chemically synthesized analogs of $1,25(OH)_2D_3$. For several key analogs a comparison of the ligand specificity has been made for the chick intestinal receptor (used in steroid competition assays) and antisera utilized for a radioimmunoassay (RIA) for plasma levels of $1,25(OH)_2D_3$. (b) We have recently developed an "exchange assay" for $1,25(OH)_2D_3$ which permits a study in the chick intestine of the relative proportion of occupied and unoccupied $1,25(OH)_2D_3$ receptors studied under a number of endocrine states. (c) We have concluded that the unoccupied intestinal receptor for $1,25(OH)_2D_3$ is largely associated with the nuclear rather than cytosol compartments. (d) We present evidence that the $1,25(OH)_2D_3$ receptor which is localized in the chromatin fraction of a low salt homogenate can exist in three different forms. These forms depend on the absence or presence of the ligand and also on whether the ligand is allowed to bind *in vitro* or *in vivo*. The unoccupied form binds the least tightly to chromatin or DNA cellulose and is therefore eluted at the lowest salt concentrations. Binding of ligand *in vitro* changes the shape of the molecule reflected in exposure of a stronger DNA binding moiety without a major change in the surface charge (evaluated by DEAE-Sepharose chromatography). Surprisingly, if the ligand is allowed to bind *in vivo*, the receptor appears in a third form with an intermediate affinity for chromatin or DNA and approximately the same apparent MW as the *in vitro* occupied form. This is the first demonstration of a heterogeneity in the $1,25(OH)_2D_3$ receptor system. References: (1) Norman, A. W. (1979) *Vitamin D: The Calcium Homeostatis Steroid Hormone*, Academic Press, New York, pp. 1-490. (2) Wecksler, W. R. and Norman, A. W. (1980) "Biochemical Properties of $1,25(OH)_2D_3$ Receptors", Steroid Biochem. 13, 977-989. (3) Walters, M. R., Hunziker, W. and Norman, A. W. (1980) "Unoccupied $1,25(OH)_2D_3$ Receptors:Nuclear-cytosol Rates Depend on Ionic Strength", J. Biol. Chem. 255, 6799-6805. (4) Walters, M. R., Hunziker, W. and Norman, A. W. (1981) $1,25(OH)_2D_3$ Receptors:Intermediates Between Triodothyronine and Steroid Hormone Receptors, Trends in Biochem. Sci. 2, 269-272. (Supported by USPHS Grants AM-09012 and AM-14,750)

0318 ORGANIZATION OF THE THYROID HORMONE RECEPTOR IN CHROMATIN, Herbert H.Samuels, Andrew J. Perlman, Bruce M. Raaka, and Frederick Stanley, NYU Med. Ctr. N.Y., N.Y. 10016. Using cultured GH_1 cells a growth hormone producing rat pituitary cell line, we have demonstrated that 3,5,3'-triiodo-L-thyronine (L-T3) stimulates a 3-to 5-fold increase in growth hormone mRNA levels (1). Multiple lines of evidence indicate that this response is regulated by a chromatin associated thyroid hormone receptor (2) which can be extracted from nuclei with 0.4 M KCl buffer (2) and is similar in properties to the receptor identified in nuclei in a variety of tissues. The salt extracted form of the receptor from GH_1 cells has a sedimentation coefficient of 3.8 S, a particle density of 1.36 g/cm^3, a Stokes radius of 3.3 nm, and an estimated molecular weight (M_r) of 54,000 (3). This M_r was further substantiated using a photoaffinity label derivitive of L-T3 in conjunction with SDS gel electrophoresis. Although these studies provide information regarding the structure of the receptor binding site they provide no information as to whether the receptor is organized as a subunit structure in chromatin since this would likely be disaggregated by high salt conditions. Studies were performed to further identify the characteristics of the chromatin associated forms of the receptor. Receptor half-life (T 1/2) and synthetic rates were determined using dense amino acid labeling techniques (4). The nuclear receptor has a T 1/2 of 4.5 h and an estimated synthetic rate of 1500 molecules/h/cell. Greater than 95% of the receptor disappears with identical kinetics indicating no major subsets of nuclear pools of receptor with regard to T 1/2. No significant pool of undetected cytoplasmic receptor was identified indicating that nuclear receptor levels are solely dependent on the rate of nuclear accumulation of newly synthesized receptor and receptor T 1/2. Micrococcal nuclease excises the receptor as a predominent 6.5 S form and as a less abundant 12.5 S species which appears to be associated with mononucleosome particles. The 6.5 S form does not bind to exogenous DNA while DNase I converts the 6.5 S species to 4.9 S and 3.8 S forms which are DNA binding species. The 6.5 S receptor has a Stokes radius of 6.0 nm, a particle density of 1.42 g/cm^3 and an M_r of 149,000. Based on the density increment of the 6.5 S vs. the 3.8 S receptor (1.42 vs. 1.36 g/cm^3), the 6.5 S receptor was calculated to be composed of a DNA component of 35-40 BP and a protein component with an M_r of 127,000. The size of the DNA component was verified by reconstitution studies with purified DNA fragments released by nuclease digestion. Only fragments in the range of 30-40 BP regenerated the 6.5 S receptor when mixed with a salt extract of nuclei. This suggests that the 54,000 M_r 3.8 S receptor may be organized as a functional complex with other protein components bound to 30-40 BP segment in chromatin (3). (1) Shapiro, L.E., Samuels, H.H., and Yaffe, B.M. (1978) Proc. Natl. Acad. Sci. U.S.A. 75:45-49; (2) Samuels, H.H. (1978) in Receptors and Hormone Action (O'Malley, B.W. and Birnbaumer, L. Eds.) Vol. 3: 35-74. Academic Press, New York; (3) Perlman, A.J., Stanley, F. and Samuels, H.H. (1982) J. Biol. Chem. In Press; (4) Raaka, B.M. and Samuels, H.H. (1981) J. Biol. Chem. 256: 6883-6889.

0319 THE PROGESTERONE RECEPTOR, William T. Schrader, Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030.

Chicken progesterone receptor has been purified from oviducts of estrogenized chicks and from laying hens. Two hormone-binding components are detected in equal amounts. Protein A (M_r=79,000) binds with high affinity to DNA whereas protein B (M_r=108,000) does not. Photoaffinity labeling of the hormone site with the synthetic progestin R5020 has been used to map this site in smaller peptides released from both A and B by chemical and enzymatic digestion. R5020-labeled fragments from the two proteins are indistinguishable by HPLC and 2-dimensional gel analysis. Thus the hormone-binding domains are very similar to each other in amino acid sequence and composition. Conversely, iodination of the two intact proteins followed by exhaustive trypsin digestion shows that the tryptic maps of tyrosyl-tryptic peptides are dramatically different. Peptides present in A are lacking in B, and the reverse. Thus, B cannot be a precursor of A. The strong DNA-binding site of A is liberated by enzymatic digestion using S. aureus V8 protease as a polypeptide (M_r=15,000) retaining the DNA-binding activity. This fragment resides on the A primary sequence adjacent to the hormone-binding domain. Similar digestions of B fail to create a strong DNA-binding sites, and thus A and B differ at this locus. We conclude that B and A are closely related distinct proteins which arise from separate cytoplasmic mRNA's, probably as products of two different genes. Proteins A and B are subunits of a larger complex, as shown by composition analysis of intact receptor aggregates, by use of bifunctional crosslinking agents and by reconstitution titration. Stoichiometry of larger complexes shows [A]= [B]; molecular weight considerations predict that the most stable conformer is an AB dimer (6S); a metastable 10S complex may be the native in vivo structure. Its composition is consistent with the formula A_2B_2. No other cofactors are presently known. Both A and B are phosphoproteins; two moles of ^{32}P can be added in vitro to each using cyclic AMP-dependent protein kinase. Neither is a substrate for myosin light chain kinase or the virus-associated kinase $p60^{src}$. Phosphorylation results in expression on both A and B of additional, weaker progesterone binding sites (K_{diss} = 25 nM) which are independent of the strong sites (K_{diss} = 0.5 nM). Dephosphorylation with alkaline phosphatase from bovine intestine destroys the weak sites but not the strong sites.

Regulatory Role of Protein Kinases

0320 THE INVOLVEMENT OF TYROSINE PHOSPHORYLATION IN THE EGF RESPONSE OF A431 CELLS, Tony Hunter and Jonathan A. Cooper, Tumor Virology Laboratory, The Salk Institute, Post Office Box 85800, San Diego, California 92138

Addition of EGF to A431 human tumor cells induces rapid phosphorylation of tyrosines present in the EGF receptor itself and in several cellular proteins. The EGF receptor, purified by immunoprecipitation with a minoclonal antibody supplied by Brad Ozanne, contains no phosphotyrosine in normal A431 cells, but becomes phosphorylated at a single site after EGF treatment. This response is maximal within two minutes. Partial sequence analysis of this site shows that there are glutamic acids 1 and 4 residues, and a lysine or arginine 9 residues upstream of the phosphotyrosine on the NH_2-terminal side. Thus this site shows strong homology with the tyrosine phosphorylation site in $pp60^{src}$ of Rous sarcoma virus (RSV), which has glutamic acids 1 and 4, and an arginine 7 residues upstream. Cohen and colleagues have shown that there is a tyrosine protein kinase in membrane preparations of A431 cells which will phsophorylate the EGF receptor in an EGF-dependent manner. One of the sites phosphorylated in this in vitro reaction is the authentic in vivo site, but the three other major in vitro sites are not phosphorylated in the cell. It appears that the in vitro system is not a faithful model for the situation in vivo.

Treatment of A431 cells with EGF causes rapid phosphorylation of an 81K and a 36K protein on tyrosine. In A431 cells infected with RSV, neither the EGF receptor nor the 81K protein is phosphorylated on tyrosine. In contrast the 36K protein, which is found to be phosphorylated in all other types of RSV transformed cell, is constitutively phosphorylated on tyrosine in these cells. Infection of A431 cells with RSV has no effect on the binding of EGF. Treatment of the infected cells with EGF leads to the phosphorylation of both the receptor and the 81K protein and an increase in phosphorylation of the 36K protein.

In collaboration with Ed Scolnick we have shown that the EGF receptor is not phosphorylated on tyrosine in A431 cells infected with Harvey sarcoma virus (HaSV) or Kirsten sarcoma virus (KiSV). Binding of EGF to these infected cells is only slightly inhibited, even though transformation of mouse cells with HaSV and KiSV has been shown to decrease EGF binding. Neither the 81K protein nor the 36K protein is phosphorylated in A431 cells infected with HaSV or KiSV, although phosphorylation of these proteins is readily detected upon treatment of the infected cells with EGF. Our data therefore suggest that neither RSV, which itself encodes a tyrosine protein kinase, nor HaSV or KiSV activate the EGF-dependent tyrosine phosphorylation system in infected A431 cells.

0321 ROLE OF EGF-DEPENDENT PROTEIN KINASE IN CONTROL OF CELL PROLIFERATION. Gordon N. Gill, Janice E. Buss, Jeffrey E. Kudlow, and Cheri S. Lazar, Department of Medicine, Division of Endocrinology, University of California, San Diego, La Jolla, CA 92093

Although EGF stimulates proliferation of many cell types, it inhibits proliferation of A431, GH_4C_1, and certain human breast cancer cells. In A431 cells which contain an unusually high density of EGF receptors, EGF increased cellular phosphotyrosine content ∿7-fold. Because EGF inhibited proliferation of A431 cells, variants resistant to this inhibition were selected after treatment with mutagen by maintenance for one month in 0.1 μM EGF. After cloning and growth for 6-20 generations without EGF, resistance to the growth inhibitory effects of EGF was confirmed. Resistant cells had diminished to no detectable increase in cellular phosphotyrosine content after treatment with EGF. Solubilized membranes from the six variants examined exhibited diminished EGF-stimulated phosphorylation of the EGF receptor and of the exogenous substrate anti-$p60^{v\text{-}src}$ IgG. The decrease in EGF-stimulated tyrosine-specific protein kinase activity varied from ∿40% in clone 16 to ∿8% in clone 18 compared to parental A431 cells. The number of EGF receptors in variant cells decreased in parallel with the EGF-stimulated kinase activity so that the specific activity of EGF kinase per EGF receptor remained constant in variants with up to 10-fold reductions in both activities. The decrease in EGF receptors was confirmed by quantitative immunoprecipitation of EGF receptors which were metabolically labeled with ^{35}S-Met using a monoclonal antiserum. Phosphorylated or metabolically labeled EGF receptors migrated identically on gels. The K_Ds of EGF binding were unaffected. A second tyrosine kinase activity, $p60^{c\text{-}sarc}$, was not altered in variants with decreased EGF-stimulated protein kinase activity. These results suggest: 1) EGF-stimulated protein kinase mediates inhibition of cell proliferation, 2) the inhibitory effect is quantitative and seen only with high levels of activated enzyme, 3) EGF receptor and kinase activity are closely coupled if not identical molecules, and 4) in conjunction with other data, EGF-stimulated kinase is distinct from $p60^{c\text{-}sarc}$.

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0322 AN EQUILIBRIUM STUDY OF THE ACTIVATION AND DEACTIVATION OF TYPE II cAMP-DEPENDENT PROTEIN KINASE AND A COMPARISON OF ITS AMINO ACID SEQUENCE WITH THAT OF cGMP-DEPENDENT PROTEIN KINASE; EVIDENCE FOR A COMMON ANCESTRAL PRECURSOR.

Stephen B. Smith*, Koji Takio+, Koiti Titani+ and Edwin G. Krebs*, Howard Hughes Medical Institute and Departments of Pharmacology* and Biochemistry+, University of Washington, Seattle, WA 98195

cAMP and cGMP-dependent protein kinases have been extensively investigated without providing conclusive answers to several important questions. Among these are the dissimilar questions, how are these kinases noncovalently regulated by their appropriate cyclic nucleotides and are these two kinases evolutionarily related? Using type II cAMP-dependent protein kinase ($R_2^{II}C_2^{II}$) from bovine cardiac tissue which was reconstituted from urea treated R_2^{II}, to remove cAMP, and native C^{II} the activation-deactivation equilibrium was studied from both directions. The cAMP activation of $R_2^{II}C_2^{II}$ was assayed by C^{II} catalyzed ^{32}P phosphorylation of Gly-Lys-Arg-Ser-Ala-Ser-Leu-Gly (PK-C), a poor substrate characterized by a K_m and V_{max} of 3.5mM and 16 mol/min/mg respectively. The large K_m value allowed kinase assays to be completed at $1.5x10^{-7}$M $R_2^{II}C_2^{II}$ which is the approximate average physiological concentration. The activation curves for phosphorylation of PK-C in response to both cAMP and ℓcAMP have been fit with the Adair equation assuming 2 and 4 site models with the best fit in each case being the 4 site model. cAMP activates the enzyme at only a 3 to 4 fold lower concentration than the analog ℓcAMP and in both cases the saturation of the first two cyclic nucleotide binding sites is a strongly positive coorperative process while the last 2 sites fill in a noncoorperative manner. The equilibrium "inactivation" of the enzyme has been studied by C^{II} titration of $cAMP_4R_2^{II}$ in the presence and absence of Mg^{2+} and ATP. Using urea pretreated R_2^{II} the following values were determined from Hill plots for the apparent equilibrium constants (K_i') for each C^{II} subunit: +Mg+ATP, $1.1x10^{-10}$,$1.3x10^{-10}$; +Mg-ATP, $1.1x10^{-9}$,$1.8x10^{-8}$; -Mg+ATP, $7.0x10^{-8}$,$2.1x10^{-7}$; and -Mg-ATP, $1.2x10^{-8}$,$5.6x10^{-7}$. From the K_i' values the equilibrium constants K_e have been calculated to be ca.: +Mg+ATP, $1.5x10^{-10}$,$1.5x10^{-3}$; +Mg-ATP, $6.8x10^{-6}$,$1.1x10^{-5}$; -Mg+ATP, $1.1x10^{-7}$,$9.6x10^{-7}$; and -Mg-ATP, $6.3x10^{-7}$,$3.5x10^{-7}$ molar, including statistical corrections. In order to ascertain the familial relationship of cAMP and cGMP-dependent protein kinases (GKP) we have completed the amino acid sequence of R^{II} and major portions of the sequence of GPK and compared these with the published sequence of C^{II}. Not only does the sequence GPK exhibit significant homology with R^{II} and C^{II} but GPK has an exposed domain that contains the autophosphorylation site and is protease labile which we termed the hinge region as in R^{II}. Both R^{II} and GPK have repeat internal amino acid homologies that suggest ancestral gene duplication. Two of the repeat sequences show a considerable degree of residue identity in both R^{II} and GPK suggesting that these sequences could have been conserved as necessary for cyclic nucleotide binding.

Receptor Mutants and Variants

0323 ISOLATION AND CHARACTERIZATION OF CHINESE HAMSTER CELL MUTANTS DEFECTIVE IN THE RECEPTOR-MEDIATED ENDOCYTOSIS OF LDL, Monty Krieger*, Michael S. Brown, and Joseph L. Goldstein, Univ. of Texas Health Science Center at Dallas, TX 75235

A two-step procedure for selection of Chinese hamster ovary cells with mutations affecting the receptor-mediated endocytosis of low density lipoprotein (LDL) will be described. In the first step, cells were incubated with reconstituted LDL containing a toxic compound, 25-hydroxycholesteryl oleats. Normal cells take up this lipoprotein via the LDL receptor pathway, liberate the toxic 25-hydroxycholesterol in lysosomes, and die. The colonies that survived this first step were then screened microscopically for LDL uptake after incubation with LDL reconstituted with a fluorescent dye. When the cells were treated with the mutagen ethyl methanesulfonate prior to selection, 1 in 10^5 cells formed colonies that survived the toxic LDL and also failed to accumulate fluorescent LDL. No such colonies were detected among 3×10^6 non-mutagenized cells. Clones derived from the mutant colonies showed absent or decreased high affinity binding of ^{125}I-LDL and were thus unable to take up and degrade the lipoprotein. Because of their receptor defect, these cells could not use LDL-cholesterol for growth or regulation of cholesterol metabolism. The mutant cells were able to grow in the presence of LDL reconstituted with 25-hydroxycholesteryl oleate at concentrations more than 100-fold above those that killed parental cells. All of these properties have been retained by the mutants for more than 175 cell doublings in the absence of selective conditions.

* Current address: Department of Biology and Whitaker College, M.I.T., Cambridge, MA 02139

0324 MUTATIONS AFFECTING THE REGULATORY SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE, Bernard P. Schimmer, Paul J. Doherty, Jennivine Tsao and Shirley A. Williams, University of Toronto, Toronto, Canada M5G 1L6

As described previously, we have isolated a family of mutant clones with defects in cAMP-dependent protein kinase (cAMPdPK) activity from ACTH-responsive Y1 adrenocortical tumor cells. We have used these mutants (designated Kin) to evaluate the importance of cAMPdPK in the various cellular responses to cAMP. Our studies have provided evidence for the obligatory involvement of cAMPdPK in the regulation of adrenal cell growth, morphology and steroidogenesis in vitro. This report explores the biochemical and molecular basis for altered cAMPdPK activity in Y1(Kin) mutant clones. Assays of cAMP-binding activity and photoaffinity labeling studies with 8-azido-cAMP suggest that the Kin mutation resides only in the Type 1 cAMPdPK isozyme. In experiments involving the reconstitution of cAMPdPK holoenzymes from isolated regulatory and catalytic subunits, the mutations in these clones were localized to the regulatory subunit of the type 1 isozyme (R^I). R^I subunits specifically immunoprecipitated from the Kin mutants had electrophoretic properties which distinguished them from the parental R^I subunit. We suggest that the electrophoretically variant forms of R^I are responsible for the altered phenotypes of the Kin mutants.

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0325 INDUCTION OF ORNITHINE DECARBOXYLASE IN MUTANTS OF CHINESE HAMSTER OVARY CELLS HAVING ALTERED CYCLIC AMP-DEPENDENT PROTEIN KINASE. J. M. Trevillyan, W. H. Fletcher & C. V. Byus. Dept. of Biochem., Div. Biomedical Sci., Univ. California, Riverside, CA 92521

The induction of ornithine decarboxylase (ODC) and subsequent accumulation of polyamines are requisite events for cell growth and proliferation. The activation of cyclic AMP-dependent protein kinase(s) (cAPK) has been proposed to mediate the induction of ODC. To better understand the relationship between activation of cAPK and the induction of ODC, the ability of 8-bromo cyclic AMP (8-Br-cAMP) to induce ODC was investigated in mutants of Chinese Hamster Ovary (CHO) cells having well characterized alterations in their cAPK [Gottesman, M. M. et al., Somatic Cell, Genet. 6, 45-61 (1980)]. 8-Br-cAMP was capable of inducing ODC in the parental cell line (CHO 10001) in a dose-dependent manner, with 1 mM 8-Br-cAMP producing a 10 fold increase in ODC activity. The induction of ODC by 1 mM 8-Br-cAMP was dramatically reduced in CHO 10260, a mutant lacking type I kinase and having less than 5% of normal levels of type II kinase; resulting in only a 3 fold increase in ODC. Similarly, 1 mM 8-Br-cAMP was incapable of inducing ODC in CHO 10215, a mutant lacking type II kinase and having an altered type I kinase. Utilizing a highly purified fluorecinated protein kinase inhibitor, free cAPK catalytic subunit was localized in CHO 10001, 10260, and 10215 before and after treatment with 1 mM 8-Br-cAMP (Byus and Fletcher, J. Cell Biol., in press). The level of increased cytoplasmic and nucleolar fluorescence correlated with the ability of 8-Br-cAMP to induce ODC in the three different cell lines. These studies are direct evidence for a role of cAPK in modulating the induction of ODC in mammalian cells.

0326 PRELIMINARY CHARACTERIZATION OF A MUTANT DEFECTIVE IN RECEPTOR-MEDIATED ENDOCYTOSIS, April R. Robbins, National Institutes of Health, Bethesda, MD 20205

Cells deficient in the uptake of two ligands, whose internalization is dependent on unrelated receptors, were isolated in order to obtain mutants with defects in receptor-mediated endocytosis subsequent to the initial step of ligand binding. Chinese hamster ovary cells were incubated with diptheria toxin, survivors were replicated and the replicate colonies were screened for their ability to accumulate radioactive proteins via the mannose 6-phosphate receptor. One of the mutants obtained exhibits many of the characteristics observed when parental (wild type) cells are treated with lysosomotropic amines: 1) While the mutant is 30 to 100 fold more resistant than the parent to diptheria toxin, resistance is abolished if, following binding of the toxin, the extracellular medium is shifted briefly to pH 4.5. This acid-induced toxicity appears to depend on the diptheria toxin receptor. 2) Endogenously synthesized acid hydrolases are secreted by the mutant in elevated amounts. 3) Functional mannose 6-phosphate receptor (measured by displacement of bound ligand) is absent or markedly decreased on the surface of the mutant, but is present on membranes prepared from lysed cells. 4) Growth of the mutant in mannose 6-phosphate or brief incubation in medium at pH 4.5, prior to measurements of binding, results in an increase in functional mannose 6-phosphate receptor on the cell surface. These results suggest that this mutant may be defective either in the translocation of ligand-receptor complexes to the lysosomes or in the maintenance of lysosomal pH.

0327 GENETIC ANALYSIS OF cAMP AFFECTS ON THE GROWTH AND THE FUNCTION OF MDCK CELLS IN HORMONALLY DEFINED MEDIUM, Mary Taub, Patricia Devis and Sue Hiller, State University of New York at Buffalo, Buffalo, New York 14214

PGE_1, a supplement in Medium K-1, the defined medium for Madin Darby Canine Kidney (MDCK) cells, has been proposed to increase both MDCK cell growth and the expression of differentiated function via a cAMP mediated mechanism(s). This hypothesis is being examined using two classes of "mutant" MDCK cells which have lesions affecting two different steps in the pathway by which PGE_1 affects MDCK cell growth and fucntion. Logarithmically growing MDCK cells have higher cAMP concentrations (and grow at a faster rate) in Medium K-1 than in Medium K-1 lacking PGE_1. Under similar growth conditions, in Medium K-1 lacking PGE_1, PGE_1 independent MDCK cells (cells which lack the PGE_1 requirement for long term growth) have a higher cAMP concentration than normal MDCK cells either in K-1 or in K-1 minus PGE_1. The increased cAMP concentration in the PGE_1 independent cells can be accounted for by an alteration in cAMP dependent phosphodiesterase. Although this lesion causes the cells to lose their growth response to PGE_1, the mutant cells still require PGE_1 to form multicellular domes (dome formation is indicative of vectorial salt and water transport). Isolated dibutyryl cAMP resistant MDCK cells have a genetic lesion which affects (a) cAMP dependent protein kinase, a subsequent step in the sequence of events initiated by PGE_1 treatment. The decrease in cAMP dependent protein kinase activity is correlated with a loss of the ability of one variant clone to make domes, although the growth response to PGE_1 is retained. These studies suggest that different kinases mediate the effects of PGE_1 on growth and domes.

0328 A GENETIC APPROACH TO STUDIES OF INSULIN ACTION IN CLOUDMAN S91 MELANOMA CELLS, J. Pawelek, M. Murray and R. Fleischman, Yale University School of Medicine, New Haven, CT 06510

Wild-type Cloudman S91 mouse melanoma cells have prolonged generation times when insulin is added to culture medium containing either horse or fetal calf sera. However, in serum-free medium the cells are stimulated to proliferate by insulin. In both cases the effects are mediated through specific high affinity insulin receptors. The effects of insulin on proliferation are dissociable from those on amino acid transport and glycogen synthesis. We isolated a number of mutants which are resistant to the inhibitory effects of insulin in serum-containing medium, and have identified three distinct phenotypes: insulin inhibited (wild-type), insulin resistant, and insulin dependent. In addition, "revertants" of the insulin dependent line were selected which no longer require insulin for proliferation. Each revertant line expressed the insulin-resistant phenotype. The insulin dependent line was about 15 times more sensitive than the wild-type to the effects of insulin, and is one of the few lines described which is stimulated to proliferate by concentrations of insulin in the 0.1-1.0 nM range. The insulin dependent line exhibited insulin-mediated protein phosphorylation patterns which differed from those seen in wild-type cells. We have produced hybrids between cells from each of the above four phenotypic categories. By testing the effects of insulin on proliferation of hybrid clones, we were able to determine that insulin inhibition is dominant over both insulin dependence and insulin resistance, and that insulin dependence is dominant over insulin resistance. Also, hybrids produced between some of our insulin resistant lines are insulin inhibited, indicating that genetic complementation occurs. Therefore, a detailed genetic analysis of the regulation of insulin action in these cells is feasible. The cloudman melanoma line represents the first system available for such studies.

Monoclonal and Other Anti-Receptor Antibodies

0329 AUTO ANTIBODIES TO THE INSULIN RECEPTOR SPONTANEOUSLY ARISE AS ANTIIDIOTYPES IN MICE IMMUNIZED TO INSULIN, Yoram Shechter, Ruth Maron, Dana Elias and Irun R. Cohen. The Weizmann Institute of Science, Rehovot 76100, Israel.

We immunized mice with insulin and found that certain strains developed anti-insulin antibodies followed by antibodies that bound to the insulin receptor and mimicked most of the actions of the hormone on rat adipocytes. In some of the sera, the insulin-like activity of the anti-receptor antibodies was equivalent to 300-400 ng insulin per ml serum. This insulin like activity was due exclusively to IgG. Anti-insulin antibodies (idiotypes) could be separated from the anti-receptor antibodies by adherence to an agarose-insulin affinity column. The anti-receptor antibodies were characterized as being anti-idiotypes because they bound to anti-insulin antibodies. Thus, an immune response induced against a hormone may lead to the spontaneous development of anti-idiotype antibodies that can interact with the hormone receptor.

0330 MONOCLONAL ANTIBODY AFFECTS NGF BINDING TO PC-12 CELLS, Linda M. Parsons, Charles E. Chandler, and Eric M. Shooter, Dept. Neurobiology, SUMC, Stanford, CA 94305

Hybridoma cells expressing monoclonal antibodies against PC-12 cells (rat pheochromocytoma) were prepared by fusing SP-2 myeloma cells with spleen cells from BALB/c mice immunized with various preparations of PC-12 cells. Spleens from mice immunized with n-octyl glucoside solubilized proteins from purified PC-12 plasma membranes generated an antibody that affected the binding of NGF to PC-12 cells; spleens from mice immunized with whole PC-12 cells or purified plasma membranes, while generating a large number of antibodies binding to the surface of PC-12 cells, did not generate any antibodies that affected NGF binding. An NGF binding assay on PC-12 cells in suspension was the screen used for detecting antibodies affecting NGF binding. Adding antibody #192 to PC-12 cells at 0° C for 1 hour prior to ligand addition (100 ng/ml 125I-NGF) doubled the binding of NGF to PC-12 cells both at 0° C (1 hr. incubation with NGF) and at 37° C (15 min. incubation), while not affecting the binding of another growth factor, EGF. At 37° C, the high-affinity NGF binding was also doubled at 15 minutes. Scatchard analysis of NGF binding to PC-12 cells at 0° C in the presence and absence of the Ab indicated an increased site number in the presence of Ab of about 60%. Adding hybridoma supernate to PC-12 cells either before or after ligand addition produced the same doubling of NGF binding. The titer of culture supernate which gave a 50% response was 1/100 when the PC-12 cell concentration was one million per ml. The Ab is an IgG that does not bind protein A. The antibody does not directly interact with NGF. Further characterization of the Ab effects should determine if it is binding to the receptor.

0331 EGF BINDING TO PC-12 CELLS IS BLOCKED BY MONOCLONAL ANTIBODY, Charles E. Chandler, Linda M. Parsons, and Eric M. Shooter, Dept. Neurobiology, SUMC, Stanford, CA 94305

We have produced monoclonal antibodies to cell surface components of PC-12 cells (rat pheochromocytoma), one of which affects the specific binding of epidermal growth factor (EGF) to PC-12 cells. Immunization of BALB/c mice with whole cells or purified plasma membrane fractions followed by spleen/myeloma (SP-2) fusions produced hybridoma cells which produced Ab to PC-12 surface components but which failed to alter EGF binding. Immunization with PC-12 octyl-glucoside solubilized plasma membrane proteins did, however, produce a hybridoma which makes Ab capable of blocking EGF binding to PC-12 cells. The screening assay involves incubating 1 vol. PC-12 cells with an equal vol. of hybridoma supernatant for 1 hour at 0° C followed by the addition of 2 vols. of 125I-EGF for an additional hour at 0° C. Final concentration of PC-12 cells is one million per ml and EGF is 60 ng/ml. Titration of the hybridoma supernatant from the active clone gives a 50% activity titer (final) of 1/50. This hybridoma supernatant has no effect on NGF binding to PC-12 cells. Activity of the hybridoma supernatant is removed by prior incubation with Staph. A cells (Pan-Sorbin). The supernatant shows a precipitin line in Ouchterlony analysis using rabbit anti-mouse IgG antiserum. The monoclonal Ab does not bind EGF directly as shown by immobilization of the Ab on a protein A coated polystyrene surface followed by incubation with 125I-EGF; therefore, the Ab is exerting its effect on the cell surface components. The monoclonal Ab also blocks EGF binding to bovine cornea endothelial cells. The Ab is more effective in blocking EGF binding when added prior to the 125I-EGF although it displaces EGF when added after 125I-EGF.

0332 MONOCLONAL ANTIBODIES TO THE HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR, Michael D. Waterfield, Elaine Mayes, Paul Stroobant, Paul Bennett, Peter Goodfellow, George Banting and Bradford Ozanne+, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A 3PX, U.K., and +University of Texas Health Science Centre, Dallas, Texas, U. S. A.

Monoclonal antibodies were raised against the human epidermoid carcinoma cell line A431 which has an unusually high number of receptors for epidermal growth factor (EGF). Following fusion of spleen cells with NSl cells, 600 clones were screened for antibodies which inhibited binding of ^{125}I-EGF to A431 cells. Six clones inhibited binding by 20-30% and one clone (R_1) was obtained as a stable line which produced large amounts of $IgG_{2\alpha}$ in ascites. Purified R_1 monoclonal did not inhibit EGF binding to A431 cells but was shown to immunoprecipitate a protein of approximately 170,000 daltons from ^{35}S-methionine labelled cells. This protein was labelled by iodination of the cell surface and could be phosphorylated by γATP in membrane preparations and in an immunoprecipitate. R_1 was able to immunoprecipitate ^{125}I-EGF (labelled with chloramine T) x-linked to a 170,000 dalton protein. Immunofluorescent studies using human fibroblast cells shows that R_1 in the presence of EGF is cleared from the cell surface. R_1 does not stimulate DNA synthesis in human fibroblast (derived) cells. (Analysis using human-mouse somatic cell hybrids and R_1 confirms the correlation of chromosome 7 with receptor expression). Taken together this evidence shows that R_1 recognises a cell surface receptor for EGF. Currently, immobilised R_1 is being used to purify this receptor for structural study by Gas-phase sequencing.

0333 MONOCLONAL ANTIBODIES AGAINST EGF-RECEPTOR INDUCE EARLY AND DELAYED EFFECTS OF EGF, by: J. Schlessinger, A.B. Schreiber, Y. Yarden and I. Lax. Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel.

Mice were immunized with human epidermoid carcinoma (A-431) cells. Spleen cells from these mice were fused with a non secreting murine myeloma (NSI cells). Several cloned hybrid lines were found to secrete antibodies (IgM) which inhibit the binding of radiolabeled EGF to membrane receptors on 3T3 and A-431 cells and human foreskin fibroblasts[1]. These monoclonal antibodies induce morphological changes in A-431 cells and enhance the phosphorylation of endogenous membrane proteins in membranes from these cells. They also stimulate DNA synthesis in human foreskin fibroblasts. Monovalent Fab fragments inhibit the binding of ^{125}I-EGF to membrane receptors but fail to induce DNA synthesis. However, cross-linking the receptor bound Fab fragments with anti-mouse antibodies leads to partial restoration of DNA synthesis. Binding studies and fluorescence microscopy studies with labeled antibodies indicate that intact anti-EGF receptor antibodies (IgM) cluster and become readily endocytosed into cultured cells while the monovalent Fab fragment are endocytosed very slowly. These observations support the notion that the biological information of the EGF-receptor complex resides in the membrane receptors and that receptor clustering could play a role in the mode of action of EGF[2].

1) A.B. Schreiber, I. Lax, Y. Yarden, Z. Eshhar and J. Schlessinger (1981) Proc. Natl. Acad. Sci. USA. December (in press)
2) J. Schlessinger (1980) Trends Biochem. Sci. 5, 210-214.

0334 MOLECULAR CHARACTERIZATION AND PURIFICATION OF β-ADRENERGIC RECEPTORS USING MONOCLONAL ANTIBODIES, Claire M. Fraser and J. Craig Venter, SUNY at Buffalo, Buffalo, NY 14214

Four monoclonal antibodies to turkey erythrocyte β-adrenergic receptors have been produced by fusion of splenic lymphocytes from Balb/C mice immunized with partially purified turkey erythrocyte β-receptors and SP2/0-Ag-14 myeloma cells using polyethylene glycol. Each monoclonal antibody was identified as an IgG molecule and it was shown that all four monoclonal antibodies precipitated soluble turkey erythrocyte β-adrenergic receptors (TEβR) in a concentration depen dent manner in an indirect immunoprecipitation assay. Scatchard analysis of saturation isotherms of IHYP binding to turkey erythrocyte membranes obtained in the presence of monoclonal antibodies indicated that one antibody (FV-101) had no effect on ligand binding to TEβR, another one (FV-103) inhibited IHYP binding to TEβR in a non-competitive fashion and a third one (FV-104) inhibited IHYP binding to TEβR in a competitive fashion. These data suggest that the antigenic determinants recognized by antibodies FV-101, 103 and 104 represent three distinct sites within the TEβR molecule. The determinant recognized by antibody FV-104 appears to be located within the ligand binding site of the receptor and this determinant is common to both β_1 and β_2-receptors from a number of sources but not to α-receptors as determined in cross-reactivity studies of ligand binding. Monoclonal antibody FV-104 was utilized in the immunoaffinity purification of β_1 and β_2-receptors. SDS-PAGE subunit compostion of purified β-receptors indicate that the β_1-receptor with a subunit MW of 70,000 and the β_2-receptor with a subunit MW of 59,000 represent distinctly different proteins.

0335 BIOLOGIC ACTIVITY OF ANTI-THYROTROPIN ANTI-IDIOTYPIC ANTIBODIES, Nadir R. Farid, Barbara Pepper, Rosario Urbina-Briones and Nazrul M. Islam, Thyroid Research Laboratory, Memorial University of Newfoundland, St. John's, Newfoundland, A1B 3V6 Canada.

An antibody against the antigen-combining site of an immunoglobulin (anti-idiotype or α-id) specific for a hormone may be active at the hormone receptor. To investigate this possibility, we immunized rabbits with rat anti-hTSH IgG purified by adsorption to TSH affinity column. Immune rabbit IgG absorbed with normal rat IgG did not bind ^{125}I b-TSH but inhibited the binding of ligand to rat anti h-TSH i.e. is α-id. The following observations suggested that α-id acted at thyroid TSH receptor: 1) It inhibited ^{125}I b-TSH binding to porcine thyroid membrane in a dose dependent manner up to 50% at 200 μg/ml compared to 16% by normal rabbit IgG (NRIgG). 2) ^{131}I α-id binding to these membranes was saturable and was inhibited by increasing concentrations of native bTSH (by 64% at 160 mU/ml). 3) Although 200 μg/ml α-id resulted in 27% reduction of thyroid membrane adenylate cyclase compared to NRIgG, when cyclase activity was assayed in the presence of 10 μM guanyl 5' - imidodiphospate (Gpp(NH)p) this concentration of α-id resulted in a 40% increase in enzyme activity over NRIgG (249 vs 356 p moles cAMP. mg protein. min.). 4) α-id caused a dose-dependent increase in the enterance rate of ^{131}I-Na compared to NRIgG into cultured thyrocytes; with a maximum of 68% at 4 hrs at 125 μg IgG/ml. 5) α-id (200 μg/ml) in contrast to NRIgG induced the organization of these cells into follicles between 5-7 days of culture.

We conclude that within the constraints imposed by TSH structure an α-id with agonist activity at the TSH receptor was made.

0336 MODULATION BY MONOCLONAL ANTIBODIES OF THE HORMONE MEDIATED GROWTH OF A LYMPHOMA CELL LINE *IN VITRO*. Juraj Ivanyi, Department of Experimental Immunobiology, The Wellcome Research Laboratories, Beckenham, Kent, UK.

Several distinct antigenic determinants were identified on native molecules of human growth hormone and chorionic somatomammotropin using murine monoclonal antibodies. The topographic relationship of these determinants to the biologically active structural moiety of the hormone was investigated by tissue culture experiments in which the extent of growth inhibition of a hormone-dependent rat lymphoma cell line (NB2) by monoclonal antibodies of distinct combining site specificity was examined. A dose-response study based on checkerboard titration of antibodies over a 10^6-fold range of dilutions against a 300-fold range of hormone concentrations indicated major quantitative differences between the respective antibodies. Some antibodies were most effective at limiting hormone doses, whereas others, although less effective at low hormone input were more potent in neutralizing excess hormone doses. The observed differences could be related to antibody specificity and affinity and various mechanisms of growth inhibition have been considered. The hormone when bound in soluble immune complexes may be prevented from binding to target cell receptors as a result of steric or conformational blocking of the hormone's active site: alternatively, antibodies may arrest the biological activity of the cell-bound hormone. The relative contribution of these factors is currently being examined.

0337 ACTIVATION OF HEXOSE TRANSPORT BY ANTIBODY, Theodore C.Y. Lo and Vincent Duronio. Univ. of Western Ontario, London, Ontario N5G 5C1

We have recently demonstrated that exposure of rat myoblast to anti-myoblast antiserum results in two to three fold activation of hexose transport. The present communication reports our finding on the possible mechanism(s) by which specific antibody can activate hexose transport in rat myoblast. Studies carried out with transglutaminase and microfilament inhibitors suggest that clustering of membrane components is essential for the activation process and microfilaments may function by bringing the membrane components together to be cross-linked by transglutaminase. The antibody-mediated activation of hexose transport is also affected by pretreatment of cells with various protease inhibitors. This suggests that protease(s) may be involved in the activation of hexose transport. In fact activation of hexose transport can be simulated by treatment of cells with low concentrations of trypsin (0.2 µg/ml). Proteolytic cleavage of cell surface components are found to occur upon incubation of cells with antibody. In view of the fact that the IgG preparation did not contain any proteolytic activity, it may be surmised that some endogenous membrane-associated proteases are activated upon binding of IgG to the cell surface. Virally-transformed myoblast was found to have elevated hexose transport activity, and anti-myoblast antiserum had no effect on this hexose transport system. This suggests that the hexose transport components in the transformed myoblasts may be present in the "active" form.

0338 MONOCLONAL ANTIBODIES AS PROBES OF THE STRUCTURE AND FUNCTION OF THE ACETYLCHOLINE RECEPTOR MOLECULE, Jon Lindstrom, William Gullick and Susan Hochschwender, Salk Institute, San Diego, CA 92138

A library of monoclonal antibodies to acetylcholine receptors from the electric organs of Torpedo californica and Electrophorus electricus and the muscle of fetal cattle and adult humans is being compiled. Thus far, nearly 200 such antibodies have been at least partially characterized. Techniques have been developed for determining the subunit specificities of those antibodies which crossreact with denatured receptor subunits (about half of the library) and for mapping the specificities of these antibodies to characteristic peptide fragments of these subunits. The binding specificities of antibodies to native receptor have been studied by competitive binding assays, formation of characteristic complexes on sucrose gradient centrifugation, binding to membrane bound vs detergent solubilized receptors, and by testing for interspecies crossreaction. The ability of antibodies to inhibit receptor function has been evaluated using carbamylcholine-induced $^{22}Na^+$ flux through reconstituted receptors. These studies have detected structural homologies between receptor subunits, defined a main immunogenic region on α subunits at which most antibodies are directed, identified a number of antigenic determinants of the α, β, γ and δ subunits of receptor, identified corresponding determinants in several species, and partially oriented some of these determinants on the amino acid sequence of the subunits and the overall structure of the molecule. Antibodies to the main immunogenic region do not impair function, but a few monoclonal antibodies have been identified which noncompetitively inhibit receptor function, and therefore bind either to the cation channel or some linkage between it and the acetylcholine binding sites.

0339 ANTIBODY INDUCED LOSS OF THE SHEEP RETICULOCYTE TRANSFERRIN RECEPTOR, B.T. Pan and R.M. Johnstone, Department of Biochemistry, McGill University.
Incubation of isolated sheep reticulocytes with rabbit antiserum prepared against sheep reticulocytes and preabsorbed with sheep mature red blood cells causes shedding or exocytosis of vesicles containing the transferrin receptor. ^{125}I-labelled, anti-reticulocyte antibody is rapidly bound to sheep reticulocytes but not to mature sheep red cells, reaching a steady state within 60 minutes at 0°C. Prolonged incubation of the reticulocytes, labelled with ^{125}I-antibody at 37°C causes a shedding or exocytosis of membrane vesicles associated with ^{125}I-labelled antibodies which also contain peptides identifiable as the transferrin receptor. This exocytosis is blocked by metabolic inhibitors, low temperatures and cytochalasin B but not by colchicine. At the protein levels detected with Coomassie Blue staining, the population of vesicles isolated appears to contain few proteins other than the transferrin receptor, transferrin and antibody. The released ^{125}I-labelled antibody associated with the vesicles no longer binds to fresh reticulocytes. It is known that the loss of glycine transport activity parallels the loss of reticulum during in vitro maturation of reticulocytes (Benderoff et al., Can. J. Biochem. 56, 545-551, 1978). To assess whether the antibody-induced loss of function is specific for the transferrin receptor or induces the loss of other cellular activities which disappear on maturation, the loss of cell-bound antibody was compared to the loss of glycine transport activity. There was no enhancement of the loss of glycine transport by the anti-reticulocyte antibody suggesting that only specific membrane proteins are shed. Supported by the MRC (Canada) B.T.P. is the holder of a University Scholarship.

Adenylate Cyclase

0340 EXPRESSION OF BETA-ADRENERGIC RECEPTORS AND ADENYLATE CYCLASE(AC) DURING THE CELL CYCLE OF S49 LYMPHOMA CELLS,Lawrence C. Mahan & Paul A. Insel, UCSD, La Jolla,CA.92093

We have used centrifugal elutriation to prepare S49 lymphoma cells located at different portions of the cell cycle and have studied cAMP accumulation and β-adrenergic receptors in these cells. Receptors were assayed on intact S49 cells using a new radioligand,[^{125}I]iodocyanopindolol(ICYP);ICYP bound to intact S49 cells with properties expected for β-adrenergic receptors with low(<10%) nonspecific binding. Receptor number/cell increased in direct proportion to the calculated increase in cell surface area during the cell cycle. Thus, the surface density of receptors/cell remains constant during the S49 cell cycle;receptor affinity for ICYP was also constant during the cycle. By contrast, β-adrenergic(isoproterenol,iso)-mediated cAMP accumulation showed cycle-specific fluctuation, being maximal in G_1 and G_2/M but lowest during S. In order to determine if these fluctuations result from changes in the catalytic unit of AC, we used the diterpene forskolin. Forskolin appears to interact with this unit in S49 cells because it activates AC in membranes from wild-type S49 cells and from an S49 variant that lacks the nucleotide coupling unit. In intact wild-type S49 cells, forskolin produces a rapid and marked increase in cAMP accumulation that is several-fold more than that produced by iso. Moreover, the forskolin-mediated increase in cAMP accumulation has a strikingly different pattern than that of iso-mediated cAMP accumulation(tested concurrently) in synchronized S49 cells studied in an experiment at 15 time points during the cell cycle. The results imply that the cycle-specific difference in expression of β-adrenergic receptors and β-adrenergic mediated cAMP accumulation cannot be explained by the expression of catalytic AC. Components of the AC complex appear to be independently regulated during the S49 cell cycle.

0341 REGULATION OF THE AFFINITIES OF THE MUSCARINIC ACETYLCHOLINE RECEPTOR IN DEVELOPING HEART FOR ANTAGONISTS AND AGONISTS BY GUANINE NUCLEOTIDES AND IONS. M. Marlene Hosey Department of Biochemistry, The Chicago Medical School, North Chicago, IL 60064.

We have previously described quantitative and qualitative differences in muscarinic cholinergic receptors (mAChR) in the developing chick heart. This report describes the regulation of the affinities of these receptors for agonists and antagonists. The antagonist radioligand [^{3}H]quinuclidinyl benzilate (QNB) was used to specifically label mAChR in homogenates of 10-day embryonic and newborn chick hearts. Depending on the conditions of the assay, three affinity states of the receptors for [^{3}H]QNB were observed: low affinity >60 pM; high affinity, 10-30 pM; and super-high affinity $\leq$ 1 pM. All assays were performed in 50 mM Tris-HCl, pH 7.4 at 37°C. In the presence of no other additions either only the high or both the high and low affinity states for [^{3}H]QNB were observed. The addition of Mg^{2+} (10 mM) and EDTA (1 mM) converted the mAChR from the high to the low affinity antagonist state. Simultaneously, the affinity for the agonist acetylcholine was increased. Guanylimidodiphosphate (GppNHp) reversed the effects of Mg^{2+}/EDTA and created mAChR with high affinity for [^{3}H]QNB and low affinity for aceytlcholine. Sodium partly reversed the effects of Mg^{2+}/EDTA and in addition created the super-high affinity state for antagonists. The data is interpreted to indicate the Mg^{2+}/EDTA uncouples mAChR from adenylate cyclase creating mAChR with high affinity for antagonists and low affinity for agonists. Guanine nucleotides and/or Na^{+} appear to promote coupling of mAChR to cyclase and create mAChR with high affinity for antagonists and low affinity for agonists.

0342 α- AND β-ADRENERGIC RECEPTORS IN THE MDCK RENAL EPITHELIAL CELL LINE, Kathryn E. Meier, Marshall D. Snavely, and Paul A. Insel, UC San Diego, La Jolla CA 92093

Madin-Darby canine kidney (MDCK) is a differentiated continuous cell line with many properties of renal tubules. We have characterized adrenergic receptors in membranes prepared from hypotonically-lysed MDCK cells. The α-adrenergic receptors were studied using the α_1-selective radioligand [^{3}H]prazosin. α-Adrenergic agonists and antagonists competed for these binding sites with relative potencies consistent with the α_1 receptor subtype. No specific binding of the α_2-selective radioligand [^{3}H]yohimbine was detected. β-Adrenergic receptors were identified using [^{125}I]iodocyanopindolol. β-Adrenergic agonists and antagonists competed for these binding sites with relative potencies typical for the β_2 receptor subtype. For both α- and β-receptors, the radioligands bound with high affinities (K_D 100 pM) to a single class of saturable sites. The total specific binding capacities represented approximately 6000 α-receptors and 4000 β-receptors per cell. Similar receptor numbers were measured in cells grown either in the presence of serum or in a serum-free defined medium. Agonists competed stereospecifically for radioligand binding to both receptors. However, agonist affinities for the β-receptor (K_D of (-)-epinephrine = 70 nM) were considerably higher than agonist affinities for the α-receptor (K of (-)-epinephrine = 5000 nM). The affinity of (-)-isoproterenol for the β-receptor was reduced 20-fold in the presence of 100 μM Gpp(NH)p. The presence of functionally coupled adrenergic receptors was demonstrated in intact MDCK cells by β-receptor-mediated cAMP accumulation and α-receptor-mediated activation of phosphatidylinositol turnover. These studies indicate that this differentiated renal cell line should be a useful model system in which to study the interplay of α- and β-adrenergic receptor systems.

0343 PROPERTIES OF SOLUBILIZED UTERINE ADENYLATE CYCLASE SUGGEST A COUPLING FACTOR-CATALYTIC SUBUNIT COMPLEX IN THE ACTIVATED STATE. Marianne Frolich, J. Frederick Krall, and Stanley G. Korenman. UCLA-SFVP, VA Medical Center, Sepulveda, CA 91343.

As with other forms of the enzyme, adenylate cyclase (AC) of a rat uterine particulate fraction was sensitive to Mg^{+2} when activated by GTP or guanylyl imidodiphosphate but to Mn^{+2} in the basal state. AC irreversibly bound [^{3}H]-GMP.P[NH]P at a rate concurrent with irreversible conversion from the Mn^{+2}- to the Mg^{+2}-sensitive form. Radioligand binding as well as activation was stimulated by isoproterenol, a further indication of binding specificity. More than 80% of Mn^{+2}-sensitive AC could be extracted from uterine homogenates in the post-100,000g supernatant (AC_{sol}) with 1% Lubrol PX. Like particulate AC, AC_{sol}) was activated by GMP.P[NH]P. Mn^{+2}-sensitive AC_{sol} sedimented as a single peak which was distinct from either of two peaks of radioligand-binding activity. AC_{sol} binding by GTP-Sepharose affinity resin and activation of particulate AC by GMP.P[NH]P-binding had the following properties in common: both proceeded at 24° but not 2°C; both were irreversible at neutral but reversible at slightly acidic pH; irreversible activation by pretreatment with GMP.P[NH]P eliminated [^{3}H]-GMP.P[NH]P binding sites and prevented binding the affinity resin. Both Mn^{+2}- and Mg^{+2}.GMP.P[NH]P sensitive enzyme activity were bound concurrently by the affinity resin, and both could be eluted with acidic buffer. We conclude: 1) Both the guanyl nucleotide-binding coupling factor (N) and the catalytic subunit (C) are extracted from uterine AC with the detergent; 2) the N- and C-subunits may be in a dissociated state in the detergent extract but; 3) form a complex to bind to the affinity resin through the N-subunit; 4) this structure might reflect a similar association in the uterine membrane.

0344 IDENTIFICATION AND CHARACTERIZATION OF α_1- AND β_2-ADRENERGIC RECEPTORS ON DDT_1MF-2 SMOOTH MUSCLE CELLS. J.S. Norris, L.E. Cornett and J.D. Garmer, Depts. of Medicine & Physiol.-Biophy., Univ. of Ark. for Medical Sciences, Little Rock, Arkansas 72205.

DDT_1MF-2 cells were derived from an androgen-estrogen induced leiomyosarcoma arising within the ductus deferens of a Syrian hamster. The cells have previously been shown to contain both androgen and glucocorticoid receptors. More recently α_1-adrenergic receptors have been identified kinetically (Cornett and Norris, JBC, in press) and by SDS gel electrophoresis of receptor labeled with ^{3}H phenoxybenzamine. This latter technique identifies a receptor with a MW of 33,000. A second specifically labeled band migrating at 16,500 is suspected to be a protein involved in Ca++ flux and has been tentatively identified as calmodulin. The β-adrenergic receptor from the DDT_1MF-2 cell line has also been examined kinetically and the data, analysed by a computer modeling technique (V.Licko, UCSF), indicate >95% pure population of β_2-adrenergic receptors. We have identified adenylate cyclase activity associated with stimulation by β-agonists and Gpp(NH)p causes a shift in the Ki of isoproterenol from 0.18±0.04μM to 0.67±0.001 μM. Propranolol, but not phentolamine, blocks epinephrine induced cAMP accumulation. These data, in conjunction with no change in the affinity of epinephrine for the α_1-adrenergic receptor in the presence of Gpp(NH)p, indicate that a functional G protein is coupled to the β_2 receptor but not to the α_1 receptor. In summary, we believe that the physiologically relevant components of a typical smooth muscle cell's adrenergic regulatory system are present in the DDT_1MF-2 cell line. Further work on these cells will help define the mechanism whereby catecholamines regulate smooth muscle cell function.

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0345 GppNHp ANTAGONISM OF THE ACTIVATION OF ADENYLATE CYCLASE BY THE DITERPENE FORSKOLIN, Kenneth B. Seamon and John W. Daly, NIADDK, NIH, Bethesda, MD 20205.
Forskolin is a potent and reversible activator of adenylate cyclase in membranes and intact cells. Activation does not require a guanine nucleotide regulatory subunit, but forskolin can potentiate hormonal responses mediated by the stimulatory (N_s) guanine nucleotide subunit. Forskolin activation can be inhibited by hormones whose effects are mediated by the inhibitory (N_i) guanine nucleotide subunit. In rat cerebral cortical membranes, GppNHp has a biphasic effect on forskolin activation of adenylate cyclase. Low concentrations of GppNHp (0.01-1 μM) inhibit the activated enzyme by 25%, while at higher concentrations of GppNHp the inhibition partially reverses. The activation by forskolin and GppNHp is never greater than that by forskolin alone. Inhibition by GppNHp is antagonized by GTP and by 10 mM $MnCl_2$. A biphasic effect of GppNHp on forskolin activation also occurs with adenylate cyclase in membranes from rat striatum, rat liver and cultured thyroid cells. All of these cyclases are subject to bimodal (stimulatory and inhibitory) regulation by hormones mediated respectively through the stimulatory N_s subunit and through the inhibitory N_i subunit. Activation of adenylate cyclase by forskolin in membranes from wild type S49 murine lymphoma cells is almost additive with GppNHp-activation. Cyclase in these membranes is not subject to bimodal regulation by hormones and thus the membranes may not contain a functional N_i subunit. It appears that GppNHp inhibition of forskolin activation of adenylate cyclase is mediated by the N_i subunit and will occur only in systems containing this subunit.

0346 CALCIUM-MEDIATED EFFECTS ON THE LIPID BILAYER OF LIVER PLASMA MEMBRANES ARE DETECTED BY HORMONE-STIMULATED ADENYLATE CYCLASE ACTIVITY AND A SPIN PROBE, L. M. Gordon, A. D. Whetton, S. Rawal, J. A. Esgate and M. D. Houslay, Rees-Stealy Research Foundation, San Diego, CA 92101. ESR studies on rat liver plasma membranes probed with 5-nitroxide stearate demonstrated that mM Ca decreased the fluidity, as indicated by increases in the order parameter (S). Over a range of concentrations similar to that which lowered the bilayer fluidity, the fluoride-stimulated (uncoupled) activity of membrane-bound adenylate cyclase (AC) was inhibited by Ca with an ID_{50} (concentration yielding 50% inhibition) of 1 mM. The fact that the fluoride stimulated activity of the membrane-bound enzyme and a Lubrol-solubilized preparation were similarly inhibited for Ca concentrations less than 2 mM suggests that Ca primarily achieves this effect not by reducing the fluidity but instead by interacting directly with either the protein or its annular lipid. The glucagon-stimulated (coupled) activity is more sensitive to Ca inhibition with an ID_{50} of 0.2 mM, and this is attributed to a direct interaction of Ca with the protein components or to structural alterations in the associated lipid, since Ca, at these low concentrations, neither inhibits the binding of glucagon to its receptor nor decreases the fluidity. Arrhenius plots of S and AC activity suggest that Ca, at mM concentrations, binds to both halves of the bilayer such that the high temperature onset of the lipid phase separation (lps) in the outer leaflet is elevated from 28° to 33°C and a second lps at 16-17°C is induced by Ca binding to acidic lipids residing in the inner leaflet. That the AC complex detects these events indicates that the actions of Ca on the lipids in the outer and inner leaflets exert significant 'second-order' perturbations on the temperature-dependence of the coupled and uncoupled activities.

0347 LIVER CYTOSOLIC ACTIVATOR OF ADENYLATE CYCLASE. H. LeVine, III, N. Sahyoun, P.J. Stenbuck and P. Cuatrecasas. The Wellcome Research Laboratories, Research Triangle Park, NC 27709.
Liver cytosol and rat or human erythrocyte ghosts were used in a reconstitution system to study soluble factors which stimulate adenylate cyclase. The resulting ten- to fifty-fold stimulation was attributed to a heat-sensitive protein which was characterized by gel-permeation chromatography and sucrose-density velocity sedimentation. This activator could interact persistently with erythrocyte plasma membranes and with cytoskeletons derived therefrom. This interaction was reversed by treatment with low-ionic strength buffer. Activation of adenylate cyclase was due to a decrease in the K_m for ATP and an increase in V_{max}. A decrease in the apparent K_m for isoproterenol was also observed. Moreover, the sequence of additions of the protein activator and of NaF, GppNHp or isoproterenol determined the extent of the resulting activation.

0348 PHOTOAFFINITY LABELLING OF THE β- ADRENERGIC RECEPTOR OF LYMPHOMA S49, Arnold E.Ruoho and Abbas Rashidbaigi, Univ. of Wisconsin, Madison, WI 53706; David Green and Richard B. Clark, Univ. of Texas, Houston, TX 77030.

The β- adrenergic receptor binding site in purified membranes obtained from cultured mouse lymphoma S49 wild type (WT) and the adenylate cyclase-deficient S49 variant, cyc⁻, has been identified using the specific photoaffinity probe, (^{125}I)-iodoazidobenzylpindolol (^{125}IABP) (Rashidbaigi, A and A.E. Ruoho, PNAS 78, 1609(1981). Photolysis of membranes from both WT and cyc⁻in the Tris-HCl, pH 7.4, 4 mM $MgCl_2$ with 1-4 nM ^{125}IABP followed by SDS gel electrophesis and autoradiography revealed the presence of two specifically derivatized polypeptides with apparent molecular weights of 55,000 and 65,000labelled in a ratio of approximately 1:1. The labelling of both polypeptides was completely prevented by either the addition of 1 uM (-)-alprenolol or 4 uM (-)-isoproterenol. ^{125}IABP labelling of both polypeptides was completely eliminated in WT after an 18-hr pretreatment with the β-agonist, terbutaline. A similar pretreatmentof cyc⁻resulted in a 50% decrease in labelling of both polypeptides. Loss of ^{125}IABP labelling was consistent with the extent of receptor loss in WT and cyc⁻measured by either epinephrine-stimulated adenylate cyclase activity or ^{125}IHYP binding. These data establish the involvement of at least two poypeptides in the β-adrenergic receptor of S49 and cyc⁻.

(Supported in part by NIH Grants NS 12392 and AM26943).

0349 THE EFFECT OF FORSKOLIN ON ADENYLATE CYCLASE OF HUMAN ADIPOCYTES AND BOVINE SPERM. D.B. Bylund, L.R. Forte, T.W. Burns and P.E. Langley, Depts. of Pharmacology and Medicine, Univ. of Missouri and V.A. Hospital, Columbia, MO 65212.

Forskolin (FSK) activates adenylate cyclase (AC) in a variety of intact cells and membrane preparations. We have studied the effect of FSK on AC in human adipocytes and porcine sperm. FSK (10 μM) increased the cAMP content of human adipocytes 100-fold and gave a greater than additive response when used in combination with isoproterenol, a beta adrenergic agonist. An alpha-2 adrenergic receptor agonist decreased both basal (30% decrease) and FSK-stimulated (90% decrease) cAMP levels. Similarly, in adipocyte membranes, FSK increased AC activity and was synergistic with respect to isoproterenol, while the FSK-stimulated activity was markedly inhibited by an alpha-2 agonist. The FSK-stimulated glycerol release was also inhibited by alpha-2 agonists. It has been reported that FSK activation does not require the GTP-regulatory protein but may directly interact with the AC catalytic unit (Seamon and Daly, JBC 256, 9799, 1981). FSK (100 μM) had no effect on sperm motility or cAMP content whereas caffeine increased both motility and cAMP content of sperm. FSK (100 μM), GppNHp and F did not significantly activate the soluble or membrane AC of sperm, whereas Mn increased AC activity. Thus, it appears unlikely that FSK directly interacts with the AC catalytic unit. The adipocyte and sperm will be useful systems for the study of the mechanism of action of FSK.

0350 AGONIST-INDUCED MODIFICATION OF β-ADRENERGIC RECEPTOR FUNCTION. J.P. Perkins, K. Harden, M. Toews, R. Doss and G. Waldo, U. North Carolina, Chapel Hill, NC 27514.

Stimulation by catecholamines of the β-adrenergic receptor (BAR)-linked adenylate cyclase (AC) in intact 1321N1 astrocytoma cells also results in agonist-specific uncoupling of the BAR/AC ($t_{1/2}$=3 min) and loss of detectable BAR ($t_{1/2}$ = 6 hr). This down regulation of cellular responsiveness appears to involve a sequence of events, $R_N \rightleftharpoons R_U \rightleftharpoons R_{LP} \rightleftharpoons R_L$, where R_N is the native BAR, R_U is an uncoupled form of BAR that binds antagonists normally but exhibits reduced affinity for agonists, R_{LP} is an uncoupled form of BAR that can be isolated on sucrose density gradients, and R_L is a form of BAR that does not bind radiolabelled antagonist ligands. When whole cell binding experiments were carried out for short periods of time (1 min) high affinity binding of isoproterenol (ISO) was observed ($K_D = 10^{-7}$M, ISO). Prior exposure of the cells to ISO, followed by washing and 1 min assays of agonist competition for ^{125}I-iodopindolol binding, demonstrated that agonists rapidly and reversibly convert intact cell BAR from a high affinity form to one of low affinity ($K_D = 10^{-5}$M, ISO). Recently we have shown that concanavalin A (250 μg/ml) blocks agonist-induced formation of R_{LP} but not R_U. Methylamine on the other hand blocks (70% inhibition at 100 mM) formation of R_L but not R_U or R_{LP}. Thus, catecholamines appear to cause rapid conversion of R_N to R_U, a form of BAR that exhibits low affinity for agonists and that cannot interact with AC. R_U is rapidly converted to R_{LP} which can be isolated in low density vesicles on sucrose density gradients and has low affinity for agonists; the formation of R_{LP} is blocked by Con A. R_{LP} appears to be converted to R_L by a process that is blocked by methylamine. The R_{LP} to R_L conversion is reversible and recovery of ^{125}I-iodohydroxybenzylpindolol binding is not dependent on dolicholphosphate mediated glycosylation or on protein synthesis.

0351 BINDING OF [^{3}H]-N-ETHYLCARBOXAMIDE ADENOSINE TO R_i ADENOSINE RECEPTORS IN RAT HIPPOCAMPUS. Richard D. Green and Helena Yeung. University of Illinois School of Medicine, Chicago, IL 60680.
The binding of ^{3}H-N ethylcarboxamide adenosine ([^{3}H]-NECA) to a crude membrane preparation of rat hippocampus has been studied. Homogenates (10 mM Imidazole Buffer, pH 7.5; 1 mM EDTA) were incubated with adenosine deaminase (5 U/ml) for 10 min at 37°C and a low speed pellet was obtained, washed 1 time in 1 mM $NaHCO_3$-1 mM EDTA (pH 7.5) and resuspended in the imidazole-EDTA buffer. Binding incubations were conducted at 37°C in the presence of additional adenosine deaminase; L-phenylisopropyladenosine (L-PIA) was used to correct for nonspecific binding. The addition of 2-8 mM $MgCl_2$ to incubation mixtures containing 1 mM EDTA increased the binding of [^{3}H]-NECA (20 nM) 5-6 fold. 4 mM $MgCl_2$ was used in subsequent experiments. Binding was antagonized by N^6-cyclohexyladenosine, 2-chloroadenosine 5'deoxy-5'-methylthioadenosine and theophylline at concentrations indicative of binding to R_i type adenosine receptors which mediate the inhibition of adenylate cyclase by adenosine analogs. The basal adenylate cyclase activity in these preparations was inhibited 20-25% by NECA or L-PIA. This inhibition was dependent on the addition of GTP and was antagonized by theophylline. Scatchard plots of specific [^{3}H]-NECA binding using concentrations ranging from approx. 3-150 nM were curvilinear and resolved into two components: K_{D_1} = 3-5 nM, K_{D_2} = 40-200 nM. Specific binding in the presence of 20 nM [^{3}H]-NECA was reduced 95% by 100µM Gpp(NH)p suggesting that both of these sites are sensitive to the presence of guanine nucleotides and thus represent high and very high affinity states of the R_i adenosine receptor. (Supported by a grant from the Campus Research Board, Univ. of Ill.)

0352 HOMOLOGOUS AND HETEROLOGOUS MECHANISMS OF ADENYLATE CYCLASE DESENSITIZATION IN HUMAN FIBROBLASTS, Peter H. Fishman and Shouki Kassis, NINCDS, NIH, Bethesda, MD 20205
Cultured human fibroblasts accumulate cyclic AMP in response to isoproterenol (ISO), prostaglandin E_1 (PGE_1) and cholera toxin (CT). Following prolonged exposure to ISO, cells became desensitized to ISO but not to PGE_1 or CT. In contrast, PGE_1-treated cells were less responsive to all 3 agents. The role of receptors (R), regulatory (N) and catalytic (C) components in homologous and heterologous desensitization was examined. Cells desensitized to ISO or PGE_1 did not lose their β-adrenergic or toxin receptors. When membranes prepared from control and desensitized cells were assayed for adenylate cyclase activities, results were comparable to those obtained with intact cells. In addition, desensitization was observed in a cell-free system and depended on GTP; kinetic parameters were similar to those observed with intact cells. When membranes were incubated with the A_1 subunit of CT and [^{32}P]NAD, 2 toxin-specific proteins of 42 and 47 kdaltons were labeled; prior desensitization by ISO or PGE_1 did not reduce ^{32}P incorporation. Cholate extracts of fibroblasts were used to reconstitute cyclase activity in membranes from S49 cyc$^-$ cells which lack N but have C. Extracts from control and ISO-treated cells were equally effective whereas extracts from PGE_1-treated cells were less effective in restoring cyclase activity. Finally, Triton N-101 extracts of fibroblast membranes were sedimented on sucrose density gradients and assayed for cyclase activity. Cyclase activity from PGE_1-treated cells sedimented at a slower rate than the enzyme from control or ISO-treated cells. We propose that ISO mediates a homologous desensitization which involves "uncoupling" of R and N and that PGE_1 mediates a heterologous desensitization which involves "uncoupling" of N and C which leads to a less efficient adenylate cyclase.

0353 UTILIZATION OF NUCLEOTIDE PHOTOAFFINITY PROBES TO STUDY ADENYLYL CYCLASE IN BIOLOGICAL MEMBRANES, Boyd E. Haley and Walid Al-Jumaily, Dept. of Biochemistry, University of Wyoming, Laramie WY 82071
Use of the photoaffinity analog [γ^{32}P]8-azidoguanosine triphosphate ([γ^{32}P]8-N_3GTP) indicates that it is a reliable probe for investigating the mechanism of action of the GTP binding protein(s) involved in the regulation of adenylyl cyclase (A.C.). 8-N_3GTP substitutes for GTP in the activation of A.C. Using 5 µM [γ^{32}P]8-N GTP and SDS-PAGE we observed photoincorporation only into a 43,000±5% MW protein of human and avian red cell membranes. Addition of GTP prevented photoincorporation. Using "time variation before photolysis" experiments we observed in situ hydrolysis of [γ^{32}P]8-N_3GTP on this protein. Washing the photolabeled membranes with various solutions allowed isolation of the labeled protein from the membrane. A comparison of [γ^{32}P]8-N_3GTP and [γ^{32}P]8-N_3ATP photoincorporation will be presented since both labeled an ≈43,000 MW protein but only [γ^{32}P]8-N_3GTP shows the in situ hydrolysis. Also, [γ^{32}P]8-N_3ATP photolabels such a species in ram sperm whereas [γ^{32}P]8-N_3GTP doesn't, and this cell type is known not to require GTP for A.C. activity. 8-N_3ATP has been observed to prevent A.C. conversion of ATP to cAMP. We therefore cautiously propose that these analogs should prove useful for identifying the GTP and ATP binding sites of A.C. as well as studying the mechanisms of hormone effects on nucleotide site occupancy. In addition, a similar 43,000 apparent MW protein was observed on isolated developing chick heart cells. Also, a protein with identical properties was found associated with Avian Myeloblastosis Virus (AMV). AMV is known to contain a very active nucleotide triphosphatase which appears to be incorporated from the host cell.

0354 MAGNESIUM TRANSPORT AND REGULATION OF ADENYLATE CYCLASE, Michael E. Maguire, Dept. of Pharmacology, Sch. Medicine, Case Western Reserve Univ., Cleveland, OH 44106

Murine S49 lymphoma cells have previously been shown to possess a β-adrenergic receptor-regulated magnesium (Mg) transport system. β-Agonists inhibit Mg influx specifically; Mg efflux is unaltered as are S49 cell transport systems for calcium, manganese, sodium and potassium. More importantly, the β-receptor-mediated inhibition of Mg influx is not mediated by cyclic AMP (Maguire and Erdos (1980) J. Biol. Chem. 255:1030). Since we have previously shown that Mg specifically activates adenylate cyclase and that the effects of β-agonists on Mg transport and adenylate cyclase desensitize independently, this suggests a role for Mg transport in the regulation of adenylate cyclase. This has been investigated further by characterizing Mg metabolism in S49 and other cells. We have now shown that a) Mg is highly compartmented, since less than 5% of total cell Mg is readily exchangeable with extracellular ion and since this small fraction forms only a small part of cytoplasmic Mg, b) free ionized intracellular Mg measured by 31-P NMR is very low, about 150 micromolar (total cellular Mg is 12 mM), c) sites for Mg interaction with the receptor-cyclase complex are localized on the cytoplasmic membrane face, and finally d) hormonal regulation of Mg transport appears to be wide-spread since we have shown hormone-sensitive Mg transport in 4 cell lines and in response to β-agonists, PGE_1, and adenosine stimulation. Taken together, these data indicate an important and probably ubiquitous role for Mg and Mg transport in regulation of the receptor-adenylate cyclase complex.

0355 ISOPROTERENOL INDUCTION OF β-ADRENERGIC RECEPTORS IN THE DEVELOPING RAT SUBMANDIBULAR GLAND, Leslie S. Cutler, University of Connecticut Health Center, Farmington, CT 06032

At birth adenylate cyclase activity in the rat submandibular gland (SMG) is not stimulated by β-adrenergic agonists. This lack of response is due to the relative paucity of β-adrenergic receptors at the secretory cell surface. Catecholamine containing neurons are absent from the gland at birth. Adrenergic nerves infiltrate the gland at 6 days after birth; the number of β-adrenergic receptors concomitantly rise and adenylate cyclase activity becomes catecholamine-responsive at this time. The purpose of the present study was to determine if there might be a causal relationship between the ingrowth of catecholamine containing neurons and the appearance of β-adrenergic receptors in the SMG. The approach taken was to determine if a β-adrenergic agonist could induce the appearance of its specific receptor in this system. Newborn Sprague Dawley rats were divided into control (saline injected) and experimental groups. Experimental animals received injections of L-isoproterenol (0.8μg/g B.W.) twice per day for 4 days. The animals were then killed by exsanguination, the submandibular glands removed and a crude plasma membrane fraction prepared. The number of β-adrenergic receptors at the cell surface was assessed by measuring [^{3}H]-dihydroalprenolol (DHA) binding. Results showed that glands from isoproterenol-treated animals had approximately 3 times more [^{3}H]-DHA bound/μg of membrane protein than glands from control animals. The K_d for both control and experimental membranes were similar, indicating no change in receptor affinity. The results suggest that the presence of the β-adrenergic agonist can induce or accelerate the appearance of specific β-adrenergic receptors at the cell surface in this developing system.

0356 EVIDENCE FOR DISTINCT GUANINE NUCLEOTIDE REGULATORY COMPLEXES MEDIATING THE STIMULATION AND INHIBITION OF ADENYLATE CYCLASE. Dermot M.F. Cooper & Donald L. Gill, National Institutes of Health, Bethesda, MD 20205

Many adenylate cyclases are subject to both stimulation and inhibition by hormone and neurotransmitters, which is governed by different ranges of GTP concentrations. Support for the distinctness of the guanine nucleotide moieties mediating stimulation and inhibition has been gathered from studies of the fat cell cyclase (e.g. Yamamura et al., 1977, J.B.C. 252, 7964; Cooper et al., 1979, J.B.C. 254, 8927) and by cholera toxin labelling studies (Cooper et al., 1981, BBRC 101, 1179). Current study of the regulation of the binding to both a stimulatory (β-adrenergic) and inhibitory (adenosine) receptor in fat cell membranes supports this notion; GTP decreases the affinity of the β-receptor in a cation independent manner; by contrast, binding to the adenosine receptor is increased by GTP in a totally Mg-dependent manner, which in turn is enhanced by sodium ion.

The effects of guanine nucleotides on binding to inhibitory receptors are currently being exploited as a probe for the integrity of N unit-receptor communication. Although catalytic activity is lost with mild NEM treatment, Ri-Ni interactions require more severe treatment for the loss of the regulation by GTP of Ri-binding. Consequently model experimental systems are available for complementation by membrane-cell fusion of systems lacking i) either Ri or ii) both Ri and Ni functions. Preliminary experiments indicate that both types of complementation are feasible.

These results are discussed in terms of distinct Ns and Ni units.

0357 ROLE OF CARBOHYDRATE IN COUPLING OF OPIATES TO THE RECEPTOR-ADENYLATE CYCLASE COMPLEX. Glyn Dawson, Ronald W. McLawhon and Clive J. Ellory.
Depts. Pediatrics and Biochemistry,Univ.of Chicago and Physiology,Univ.of Cambridge,U.K.

$D_{Ala^2}D_{Leu^5}$ Enkephalin binds to membrane preparations from neurotumor cell lines N4TG1, NG-10815 and NCB-20 with nanomolar affinity. Radiation inactivation analysis suggest a receptor molecular weight of 750,000 for agonist binding but only 250,000 for antagonist (naloxone) binding. Agonist binding is inhibited by Na^+ and guanyl nucleotides but stimulated slightly by divalent cations such as Mn^{2+}. Coupling of opiate agonists to the receptor activates GTPase, inactivates adenylate cyclase, protein kinase and a number of glycolipid (and possibly glycoprotein glycosyltransferases). If carbohydrate has an important role in binding enkephalin then such a process could explain receptor desensitization. Tunicamycin (0.15 to 0.3 g/ml for 24h) inhibits the addition of N-linked oligosaccharide units to glycoproteins. Under conditions where [^{3}H]glucosamine incorporation was inhibited 89% and [^{14}C]leucine incorporation less than 5%, the number of opiate binding sites decreased by more than 50%. The loss was not reversed by adding protease inhibitors and it was not possible to release Golgi- sequestered receptors. Tunicamycin treatment also induced a supersensitivity to Mn^{2+} and the residual binding could not be inhibited by GTP. Neuraminidase treatment did not significantly inhibit binding but prior incubation with various lectins did. These data suggest that the enkephalin receptor is a large glycoprotein complex whose conformation is regulated by a number of factors including oligosaccharide units, nucleotides and metal ions. (Supported by USPHS, HD 06426, 09402 and DA02575.

Receptors for Toxins and Toxin Hybrids

0358 MECHANISM OF ACTION OF THE EDEMA FACTOR OF ANTHRAX TOXIN, Stephen H. Leppla, US Army Med. Res. Inst. Infec. Dis., Frederick, MD 21701

Anthrax toxin consists of three proteins which individually cause no toxic effects. The combination of protective antigen (PA) with lethal factor (LF) kills rats in 60 min, and PA combined with edema factor (EF) causes edema in the skin of rabbits and guinea pigs. These three proteins were purified to near homogeneity from culture supernatants of the avirulent Sterne vaccine strain of B. anthracis. The mechanism of EF was studied. EF combined with PA caused elongation of Chinese hamster ovary (CHO) cells, which was shown to result from dramatic elevations in cAMP concentrations. Intracellular cAMP in treated cells rose without a lag to concentrations 500-fold above normal values, remained high in the continued presence of toxin, and fell rapidly after toxin removal. Other cultured cell lines also showed elevated cAMP when treated with PA and EF. This response was prevented by excess LF. These results suggest that PA binds to cells and forms an uptake system that can be used by either EF or LF.

Edema factor was found to be an adenylate cyclase which requires both calcium and a heat stable eukaryotic cell material, probably calmodulin. Excess calcium (1mM) inhibits in the presence of 10 mM Mg^{++}, but not in 10 mM Mn^{++}, as has been shown for the calmodulin-dependent brain adenylate cyclase. EF is the first adenylate cyclase isolated from the genus Bacillus. Its specific activity, 20 μmol cAMP min^{-1} mg^{-1}, exceeds that of any other known cyclase. The similarity to brain adenylate cyclase suggests a common evolutionary origin.

0359 MONENSIN BLOCKS THE TRANSPORT OF DIPHTHERIA TOXIN TO THE CELL CYTOPLASM, Rockford K. Draper and Mary H. Marnell, The University of Texas at Dallas, Richardson, TX 75080

Lysosomotropic amines are believed to inhibit the transport of diphtheria toxin to the cell cytoplasm by raising the pH within intracellular vesicles. If so, then other drugs that dissipate intracellular proton gradients should have a similar effect on toxin transport. We found that monensin, a proton ionophore unrelated to lysosomotropic amines, is a potent inhibitor of the cytotoxic effect of diphtheria toxin. Monensin appears to block the escape of endocytosed toxin from a vesicle to the cytoplasm. Monensin fails to protect cells from the effects of diphtheria toxin that is bound to the cell surface and exposed to acidic medium, suggesting that the step normally blocked by the drug is circumvented under these conditions. The inhibition of toxin transport caused by monensin could not be relieved when monensin was replaced by ammonium chloride, nor when ammonium chloride was again replaced by monensin. This suggests that both drugs block the same step of toxin transport. The effect of monensin on the transport of diphtheria toxin to the cytoplasm is consistent with the proposal (Draper and Simon, 1980, J. Cell Biol. 87, 849-854; Sandvig and Olsnes, 1980, J. Cell Biol. 87, 828-832) that the toxin is endocytosed and then, in response to an acidic environment, penetrates through the membrane of an intracellular vesicle to reach the cytoplasm.

0360 INHIBITION OF MEMBRANE ASSOCIATED METABOLIC PROCESSES BY LEGIONELLA PNEUMOPHILA TOXIN, Janis E. Lochner, Richard L. Friedman, Barbara H. Iglewski, Robert H. Bigley, and Libuse Stankova, Oregon Health Sciences University, Portland, Ore. 97201

The potent antimicrobial activity of polymorphonuclear leukocytes (PMN) derives primarily from the ability of these cells to generate toxic oxygen metabolites. Upon interaction with appropriate stimuli, a membrane associated superoxide anion (O_2^-) generating complex is activated. Accompanying the activation of this enzyme complex are abrupt increases in O_2 consumption, O_2^- and H_2O_2 production, and NADPH production via the hexose monophosphate shunt (HMPS). Legionella pneumophila, the causative agent of Legionnaires' disease, manages to evade the antimicrobial system of PMN and to survive intracellularly. A low molecular weight toxin produced by L. pneumophila, which is toxic to a variety of tissue culture cell lines, has been identified. We have attempted to discern if this toxin is involved in the ability of L. pneumophila to survive intracellularly. Initially, we observed that a one hour incubation of PMN with L. pneumophila toxin dramatically inhibited phagocytosing O_2 consumption and HMPS activity in a dose dependent manner, while exerting no influence on the phagocytic ability of the cells. The HMPS activity of toxin treated PMN could, however, be stimulated by methylene blue, which indicated that the integrity of the HMPS had not been perturbed and suggested that the toxin was interacting with either the PMN membrane or a component of the superoxide anion generating complex. In later studies, we demonstrated that the ability of toxin treated PMN both to iodinate and to kill bacteria was also significantly impaired.

0361 CELL SURFACE RECEPTORS FOR CYTOLYTIC TOXINS, W. T. Shier, D. J. DuBourdieu and H. Kawaguchi, University of Minnesota, Minneapolis, MN 55455.

We have identified a class of cytolytic toxins which appear to act by the following mechanism: (1) binding to cell surface receptor sites; (2) activation of cellular phospholipase A_2 with production of lysolecithin and free fatty acids; (3) altered membrane permeability to Na^+ ions with Na^+ influx; and (4) osmotic lysis. A subclass of these toxins (e.g., the toadstool toxin, phallolysin) has been identified which binds to sites on Swiss 3T3 mouse fibroblasts similar to or identical with wheat germ agglutinin (WGA) binding sites. A new member of this subclass has been identified in the venom of a sea anenome (Condylactis gigantea), which activates high levels of cellular phospholipase (hydrolysis of up to 55% of labeled lipids in 30 min) and prostaglandin E_2 synthesis (conversion of up to 5.3% of ^{3}H-arachidonate released) in 3T3 cells biosynthetically labeled with ^{3}H-arachidonate. Of a series of potential phospholipase inhibitors only p-bromophenacylbromide (0.1 mM) inhibited the phospholipase. Activation of phospholipase by condylactis toxin, but not by a control (Portuguese Man-'O-War toxin), is inhibited by pretreatment with WGA (100 µg/ml, 20 min., 37°) but not with soybean agglutinin. A variant 3T3 cell line selected for resistance to phallolysin exhibited cross-resistance to toxicity by WGA and by condylactis toxin but not by Portuguese Man-'O-War toxin. Studies on inhibition of phallolysin- and condylactis toxin-induced hemolysis and WGA-induced hemagglutination by a series of saccharides demonstrated similar but distinctly different carbohydrate specificities for the 3 agents. Supported by NSF grant PCM 80-11784.

0362 [^{125}I] IODOESTRADIOL IS SPECIFICALLY CYTOTOXIC FOR ESTROGEN RESPONSIVE BREAST CANCER CELLS, Marc E. Lippman, D.A. Bronzert, R.A. Hochberg, Medicine Branch, NCI, Bethesda MD and Dept of Obstetrics and Gynecology, Yale University School of Medicine, New Haven CT

It has previously been shown that ^{3}H-labeled thymidine is cytotoxic to replicating cells when incorporated into their DNA. We asked whether this technique could be used to specifically kill cells containing estrogen receptors. MCF-7 cells are incubated in 8×10^{-10}M 16 [^{125}I] Iodoestradiol (>1600Ci/mmole) (IE_2) with and without 100-fold excess cold estradiol (E_2) for 1 hour at 37°C. The cells are then washed in the above media to remove excess nonspecifically bound steroid and viably frozen in the above medium plus 5% DMSO. The final concentration of radioactivity in the MCF-7 cells was 0.173 DPM/cell for those exposed to IE_2 alone and 0.026 DPM/cell for those exposed to IE_2 plus E_2. The difference represents specifically bound receptor associated estradiol. The cells are defrosted at various times and allowed to grow for 4 to 5 divisions. These cells are harvested and counted. The MCF-7 cells incubated with IE_2 are 54% of controls (cells incubated with the ethanol vehicle and frozen). Those cells incubated with IE_2 plus E_2 are 110% of control. After 8 weeks, the IE_2 cells and IE_2 plus E_2 cells are 10% and 77% respectively. A breast cancer cell line which does not contain estrogen receptor (MDA-231) was also used as a control. When defrosted after freezing, their rate of cell death as a function of freezing time was similar to the MCF-7 cells treated with radioactive estradiol plus competitor. Using an alternative cloning assay, IE_2 induces a 5 log reduction in receptor containing cells. This technique shows great promise in selecting a population of cells with defects in their estrogen receptor and in studying subcellular hormone interactions.

0363 THE BIOLOGICAL ACTIVITY OF INSULIN-CHOLERA TOXIN B CHAIN CONJUGATE, R.A. Roth, B.A. Maddux, and I.D. Goldfine, Cell Biology Laboratory and Department of Medicine, Mount Zion Hospital and Medical Center, San Francisco, CA 94120

Insulin binds to specific receptors on the plasma membrane of target cells and then elicits its biological effects. To probe the role of the receptor in this process, we synthesized a hybrid molecule composed of insulin linked to the binding subunit of cholera toxin via a disulfide bond. This conjugate has the potential to bind to cells either via insulin receptors or via the cholera toxin receptors, the GM_1 gangliosides. That the insulin-cholera toxin B chain conjugate interacts with gangliosides was shown by the ability of the conjugate to bind to a ganglioside-sepharose column. The interaction of the conjugate with insulin receptors was examined by testing its ability to inhibit the binding of ^{125}I-insulin to its receptor on IM-9 human lymphocytes. The conjugate was found to have 1/20 the ability of native insulin in binding to the insulin receptor. The conjugate was then examined for insulin-like biological activity by testing its effect on AIB uptake into rat thymocytes. The conjugate had 1/2 the biological activity of native insulin, a value 10-fold greater than its ability to bind to the insulin receptor. In contrast, uncoupled cholera toxin B chain had no effect on AIB uptake and did not influence stimulation by native insulin. To assess the contribution of the cholera toxin B chain to the biological activity of the conjugate, gangliosides were added with the conjugate. At 3 µg/ml, GM_1 ganglioside inhibited the activity of the conjugate by 50% without effecting the activity of native insulin. These results suggest, therefore, that coupling insulin to the cholera toxin B chain produces a biologically active insulin molecule with new receptor specificity.

0364 TOXIN-HORMONE CONJUGATES AND RESISTANT VARIANTS, N. Shimizu, W.K. Miskimins, S. Gamou and Y. Shimizu, Dept. Cell. & Dev. Biol., Univ. of Arizona, Tucson, AZ 85721

We previously isolated several variants of mouse Swiss/3T3 fibroblasts which are resistant to a cytotoxic insulin-diphtheria toxin A fragment conjugate (BBRC 91:143, 1979; PNAS 78:445, 1981). Insulin binding ability of these variants were either deficient or substantially reduced and one variant accumulated lysosome-like vesicles (JSSCB 16:105, 1981). Recently we have studied the insulin binding surface components of the variants which display reduced insulin binding activity using an affinity crosslinking method followed by polyacrylamide gel electrophoresis. The analysis revealed that in parental 3T3 cells three major membrane components (Mr=135K, 100K and 70K) are readily affinity crosslinked to ^{125}I-insulin while in the variants CI-3 and CI-7a only two major components (Mr=135K and 70K) are affinity crosslinked. There were multiple forms of large molecular weight ^{125}I-insulin binding components in these cell lines. Considering the recent proposal for insulin receptor structure by Czech et al. (PNAS 77:7137, 1980), the Mr=135K component may be a large (α) subunit of the insulin receptor and the Mr=100K component a small (β) subunit. The variants, CI-3 and CI-7a, may possess a mutation in the gene for the small subunit. Alternatively, the β subunits are produced in these variants but are somewhat inaccessible to affinity crosslinking. As an extension of our genetic approach, we have produced an insulin-ricin A conjugate and applied it for isolating genetic variants of 3T3-L1 preadipocytes (CSHC 9:74, 1981). Similar conjugates were made using epidermal growth factor and toxic DTa or ricin A (FEBS Lett. 118:274, 1980). Because of differences in the nature of crosslinking, comparative studies are being conducted in terms of cell killing profile, inhibition of protein synthesis and receptor specificity.NIHGM24375 ACSIFRAG

0365 ESTROGEN RECEPTOR-MEDIATED CYTOTOXICITY USING IODINE-125, W.D. Bloomer, W.H. McLaughlin, R.R. Weichselbaum, and S.J. Adelstein, Harvard Medical School, Boston, MA 02115

There is abundant evidence that Auger effects from ^{125}I are singularly damaging if localized within DNA as the thymidine analogue ^{125}I-iododeoxyuridine (^{125}IUdR).

We evaluated the cellular uptake of iodine-125 labeled tamoxifen (^{125}ITAM) and its in vitro cytotoxicity by a clonal survival assay in MCF-7 (ERP=60 fmol/mg) and V-79 (ERP=4 fmol/mg) cells. ^{125}ITAM was differentially cytotoxic to MCF-7 cells; the D_{37} values were 0.5 and 1.5 pCi/cell for MCF-7 and V-79 cells, respectively. Non-radioactive ^{127}ITAM, TAM and Na^{125}I were all non-toxic at levels of toxicity observed with ^{125}ITAM.

We compared the relative cytotoxicities of ^{125}IUdR, and ^{125}I-iodoantipyrine, a biological indicator of the body water space, in MCF-7 cells. Cytotoxicity was dependent upon subcellular localization. When survival was expressed as a function of the concentration of ^{125}IUdR, ^{125}ITAM and ^{125}IAP in the culture media, the D_{37} values were 8×10^{-4}, 2.3 and 68 µCi/ml, respectively. However, when survival is expressed as a function of the nucleic acid and protein subcellular fraction, ^{125}ITAM is just about as toxic as ^{125}IUdR localized within the DNA backbone. The fact that the two survival curves cannot be superimposed probably reflects contributions of extranuclear ^{125}ITAM bound non-specifically to cytoplasmic protein as well as differences in genome associations and nuclear retention time. The differential cytotoxicity of ^{125}ITAM in cells containing estrogen receptor affords a new approach to the study of subcellular hormone interactions and suggests a methodology for using iodinated hormones and their analogues as diagnostic agents and carriers of radionuclides for therapy.

0366 SELECTIVE CYTOTOXICITY MEDIATED BY THE ESTROGEN RECEPTOR, Benita S. Katzenellenbogen, Lisa L. Wei, David W. Robertson, and John A. Katzenellenbogen, Departments of Physiology and Chemistry, University of Illinois, Urbana, IL 61801

The estrogen receptor of target tissues and tumors provides a mechanism for the cellular concentration of estrogens or antiestrogens. Hence, it should be possible to effect a selective, receptor-mediated killing of estrogen receptor-rich tumor cells with a suitable cytotoxic estrogen derivative. We have prepared and studied a series of derivatives of the antiestrogen tamoxifen embodying reactive and potentially cytotoxic functions (aziridine, nitrogen mustard, nitrosocarbamate and nitrosourea groups), and we have assessed these compounds in terms of their interactions with receptor and their toxicity towards receptor positive and negative cells. The tamoxifen aziridines have a good affinity for the estrogen receptor, react covalently with the estrogen receptor, and they are effective inhibitors of the growth of MCF-7 human breast cancer cells and DMBA-induced rat mammary tumors. The nitrosourea compound also inhibits cell growth by an estrogen receptor-mediated process. These compounds should be useful for studying estrogen receptor dynamics in cells and may have enhanced antitumor activity.

Receptors for Glycoproteins

0367 EVOLUTION OF CARBOHYDRATE-BINDING PROTEINS: POTENTIAL RELATIONSHIP OF DISCOIDIN I AND CHICKEN HEPATIC LECTIN. William A. Frazier, Washington Univ., St. Louis, MO 63110.

The slime mold lectin discoidin I has a subunit M_r of 28,000, binds N-acetyl-galactosamine and peripherally associates with membranes. The chicken hepatic lectin has a M_r of 26,000, binds N-acetyl-glucosamine and is an integral membrane protein. Both are rich in Cys and Trp. Nucleotide sequence analysis of discoidin I genes has indicated that four closely related genes comprise a multigene family and the amino acid sequences of 4 discoidins I have been deduced (Poole et al., J. Mol. Biol., in press). Internal homology within the discoidin I sequences indicates that the present discoidin I genes have arisen by contiguous duplication of a gene coding for a 155 residue protein followed by deletion of a segment corresponding to 60 amino acids in the C-terminal duplicate. This internal alignment shows conservation of 6 of the 8 half-cystines and 2 of the 5 tryptophans. The gene coding for the 253 residue discoidin sequence was then duplicated four times giving rise to the present multigene family. Comparison of the discoidin I sequences with that of the chicken hepatic lectin (Drickamer, J. Biol. Chem. 256, 5827, 1981) suggests a distant but significant relationship. The alignment of discoidin I with hepatic lectin yields 25% identities, many of which are found clustered near conserved cysteines and tryptophans. This potential evolutionary relationship of slime mold and chicken lectins has interesting implications both for the function of each lectin and for the evolution of membrane receptors for glycoconjugates. (Supported by NIH and NSF. WAF is an Established Investigator of the American Heart Association.)

0368 CHARACTERIZATION OF ERYTHROPOIETIN BINDING TO MARROW CELLS. Tania L. Weiss and Eugene Goldwasser, University of Chicago, Chicago, IL 60637.

Since ^{125}I-erythropoietin, the primary inducer of red blood cell development, is biologically inactive, we have used a monoclonal antibody to erythropoietin (epo) and an active fluorescent epo adduct, N-(7-dimethylamino 4-methylcourmarinyl-)maleimide-epo (DACM-epo), to study its binding to marrow cells. Recently we have produced a monoclonal antibody which is specifically directed against epo and does not inactivate it. It is an immunoglobulin of the IgG class which binds the α and β forms as well as the asialo α form to the same extent. Using fluorescence and phase optics microscopy we have determined the total number of live rat and mouse bone marrow cells which bind epo in 20 to 60 fields containing 1,000 to 6,000 cells. With indirect immunofluorescence microscopy we detect 1.3% positive cells over controls (P .001) when mouse marrow cells are incubated one hour with epo at 37°C. The number of CFU-e found was 0.55%. With direct fluorescence microscopy when DACM-epo is incubated with rat marrow cells for one hour at 37°C., 1.4% positive cells over the controls (P .001) was obtained, a close correlation with the indirect immunofluorescence data obtained in mouse marrow cell cultures. DACM-epo binding was successfully competed with by a 100 fold excess of native epo and no binding was observed in one hour of incubation at 4°C. Thymus cells, erythrocytes and Friend erythroleukemia cells showed no binding in one hour at 37°C. DACM-epo binding is responsive to the extent of erythropoiesis within the marrow in that hyperplastic marrow showed 5% positive cells (P .001), plethoric marrow showed 0.8% (P .05). Hypoxic marrow was not different from controls. (Supported in part by Grants HL 21676 and HL 1600 from the NIH)

0369 THE HEPATIC ASIALOGLYCOPROTEIN RECEPTOR IS NOT INVOLVED IN CLEARING THE RAT'S BLOODSTREAM OF INTACT GLYCOPROTEINS, Rudolf Clarenburg, Kansas State University, Manhattan, KS 66506.

The hypothesis that the hepatic receptor for asialoglycoproteins would mediate turnover of glycoproteins in blood (via prior desialylation by neuraminidase activity located on cell surfaces) was tested in rats by measuring the clearance of intact ^{125}I-labeled glycoproteins in the presence, or not, of enough unlabeled asialoglycoproteins to saturate the asialoreceptor. Via an indwelling venous catheter, a buffered solution containing ^{125}I-orosomucoid (obtained from rat serum) and bolus of unlabeled asialofetuin (bovine) was injected, followed immediately by an infusion of an asialofetuin solution. Total radioactivity, and trichloroacetic acid-precipitable radioactivity, were measured in blood samples taken at frequent intervals from an arterial catheter. Removal of radioactivity from blood was computer-analyzed on the basis of a logarithmic model with two components defined by their halflives and ordinate intercepts. To demonstrate the nondestructiveness of radiolabeling and desialylation methods used, and the interchangeability of rat and bovine (asialo)glycoproteins, ten groups of control experiments were needed in which labeled (asialo)fetuin or (asialo) orosomucoid were injected in the presence, or not, of bolus and infusate of unlabeled (asialo) glycoproteins. The results of those clearance trials showed that intact- and asialo-glycoproteins are removed from blood by independent processes. Measuring the distribution of ^{125}I-fetuin or ^{125}I-asialofetuin in different tissues of the rat at various times after injection corroborated the conclusion that intact- and asialo- glycoproteins have different routes of metabolism.

0370 REDUCED GLYCOPROTEIN RECEPTOR ACTIVITY IN DIABETES. Hak-Joong Kim, and Indira V. Kurup, Medical College of Wisconsin, Milwaukee, WI 53226

Recent reports demonstrated that the plasma and tissue proteins could be nonenzymatically glycosylated on its E-amino group of lysyl residues by persistent hyperglycemia in diabetic subjects, and it could contribute to diabetic complications. However, the catabolic pathways of these proteins are poorly understood. Since asialoglycoprotein recognizing receptors (ASGR) in mammalian liver do not differentiate between galactose and glucose terminated glycoprotein, the catabolism of glycosylated human albumin (gAlb) and low density lipoprotein (gLDL) were studied in normal human fibroblasts,and in vivo in rats, and compared with that of the normal albumin (nAlb) and normal LDL (nLDL). Albumin or LDL was purified by ion exchange chromatography or by ultracentrifugation, and was labeled with I^{125} by iodine monochloride technique. Aliquots were glycosylated by reductive amination using glucose or by imidination using thioglucoside (Lee et al. 1974). When more than 50% of the lysyl residues were modified gLDL did not bind to and was not catabolized by nLDL receptors in normal human fibroblasts. When 50-150 µg was injected i.v. the gLDL was metabolized with a plasma half life less than 10 min compared to 6-7 hrs for nAlb or nLDL. The accelerated removal of these proteins was partially blocked by simultaneous injection of asialofetuin. While the catabolic rate of gAlb or gLDL was significantly reduced in severely diabetic rats ($T^1/_2$= 2 hr), the turnover rate of nLDL or nAlb was not altered. These data suggest that ASGR may play an important role in the catabolism of glycosylated plasma proteins, and its activity is reduced in diabetic animals, contributing further accumulation of these proteins in plasma.

0371 SPECIFIC DEFICIENCY OF A PLATELET MEMBRANE GLYCOPROTEIN ASSOCIATED WITH DEFECTIVE VON WILLEBRAND FACTOR BINDING. Dolores M. Peterson, U of TX Med School, Houston, TX 77030.

Platelets from patients with Bernard-Soulier (B-S) disease, an inherited bleeding abnormality, fail to bind von Willebrand factor (Thromb. Res.19:21,1980) and exhibit defective adhesion to subendothelium. In an attempt to identify the defective receptor in B-S platelets, platelets from normal individuals and from four patients with Bernard-Soulier disease were radiolabeled on the surface by lactoperoxidase-catalyzed (^{125}I)iodination or by the periodate-(^{3}H)borohydride reaction. The platelet protein and glycoprotein compositions were then analyzed by specific staining and autoradiography after separation by the O'Farrell two-dimensional gel electrophoresis technique. In normal platelets, five periodic acid-Schiff (PAS) stained glycoproteins were resolved in the glycoprotein I (GP I) molecular weight region of the two-dimensional gels. These glycoproteins were designated Ia (M_r=158,000 and pI of 5.33), Ic (M_r= 131,000 and pI of 5.36), IIa (M_r=146,000 and pI of 4.65), Is/Ib (M_r=139,000 and pI of 6.36), and Id (M_r=134,000 and pI of 5.91). Bernard-Soulier platelets contained four of the glycoproteins of the GP I complex but were deficient in the glycoprotein Is/Ib which, in normal platelets, was not stained by Coomassie blue but had a high affinity for both PAS-stain and alcian blue stain and was heavily labeled on the surface of platelets by the periodate-borohydride reaction but only faintly with radioiodine. Is/Ib was not detected in membranes isolated in presence of 1mM EDTA suggesting that Is/Ib is a loosely-attached outer-coat protein, and the absence of this protein in B-S platelets suggests that Is/Ib is the platelet receptor for von Willebrand factor.

0372 UPTAKE OF MANNOSE TERMINATED GLYCOCONJUGATES BY MACROPHAGES IN INHIBITED BY AMMONIUM CHLORIDE, MONENSIN AND METABOLIC INHIBITORS, Philip Stahl, Teri Takehiro, Rita Boshans, Chris Tietze and Paul Schlesinger, Dept. of Physiology, Washington University Medical School, St. Louis, MO 63110

Mannose-terminated glycoproteins and various lysosomal enzymes are recognized by pinocytosis receptors on macrophages. Alveolar macrophages have about 75,000 high affinity binding sites on their surfaces yet the cells can take up 2 x 10^6 molecules/hour (Stahl et al., Cell 19: 207, 1980). Receptor recycling or reutilization is the likely explanation for these data. Amines inhibit ligand uptake but not ligand binding (Tietze et al., BBRC 93: 1, 1980). The amine-induced block appears to be beyond the internalization step and is dependent upon pH. These results suggest that acidification of an intracellular vesicle containing the receptor-ligand complex is required for receptor reutilization. Monensin appears to act similarly to amines. Inhibition of ^{125}I-β-glucuronidase uptake into macrophages by monensin is concentration-dependent with 50% inhibition produced by 10^{-6}M monensin. Fluoride and sodium vanadate are effective inhibitors of uptake whereas azide and CCCP were found to be poor inhibitors.

Role of Intracellular Peptide Hormone Receptors

0373 COATED VESICLES FUNCTION IN THE RECEPTOR-MEDIATED ENDOCYTOSIS OF INSULIN, Paul F. Pilch, Michael A. Shia and Richard E. Fine, Boston University School of Medicine, Boston, MA 02118.

Insulin bound to cell surface receptors can undergo several possible fates including internalization and degradation in cellular lysosomes. Receptor mediated endocytosis via coated pits and coated vesicles (CV's) have been implicated in this process. Rat liver posesses the capacity to internalize and degrade insulin. We have purified CV's from rat liver by differential ultracentrifugation to greater than 95% homogeneity as determined by electron microscopy and agarose and polyacrylamide gel electrophoresis. We have demonstrated that these rat liver CV's exhibit a latent insulin binding capability. In the absence of detergent, no specific binding of ^{125}I-insulin can be demonstrated. However, CV's treated with the detergent octyl glucoside exhibit substantial specific insulin binding. The insulin binding protein present in CV's has been specifically labelled by linking ^{125}I-insulin to detergent treated vesicles using the bifunctional crosslinking reagent disuccinimidyl suberate. Electrophoretic analysis revealed that the labeled protein migrated in an identical fashion to the rat liver plasma membrane protein previously identified as the high affinity insulin receptor. Hepatic portal vein injection of insulin followed by liver excision and coated vesicle isolation revealed that insulin was taken up into coated vesicles. The time course and specificity of insulin uptake into coated vesicles after hepatic portal vein injection of hormone is consistent with a role for coated vesicles in the receptor-mediated endocytosis of insulin.

0374 INTERNALIZATION AND RECYCLING OF INSULIN RECEPTORS IN ISOLATED RAT HEPATOCYTES; M. Fehlmann, J.L. Carpentier, E. Van Obberghen, D. Brandenburg, L. Orci and P. Freychet; Inserm U145, Med. School, Nice, France; Dept. of Morphology, Med. School, Geneva, Switzerland, and Deutsches Wollforschungsinstitut, Aachen, FRG.

This study was undertaken to investigate the fate of insulin receptors following the initial binding of insulin. Cell surface insulin receptors of isolated rat hepatocytes were specifically labeled at 15°C with a ^{125}I-photoreactive insulin analogue covalently coupled to the 130K receptor subunit by UV-irradiation. Hepatocytes were then incubated at 37°C and the cellular localization of the radioactivity was followed by analyzing the distribution of autoradiographic grains after electron microscopy. Within 60 min 80% of the grains were found inside the cells, predominantly associated with lysosomes. Under these conditions SDS-PAGE analysis showed that most radioactivity was still associated with an intact 130K protein species. Moreover limited exposure of hepatocytes to trypsin at the end of the incubation of 37°C did not affect the labeling pattern of the insulin-receptor complexes on SDS gels, further indicating that the insulin receptor was internalized with insulin. However, increasing periods of incubation at 37°C resulted in a progressive return of autoradiographic grains to the hepatocyte plasma membrane (60% of the grains distributed around the plasma membrane after 300 min) and concomitantly SDS-PAGE showed that the 130K insulin-receptor complexes became progressively more sensitive to trypsin. These results thus demonstrate that in hepatocytes insulin receptors are internalized with insulin and are progressively recycled to the cell surface.

0375 ENDOCYTOSIS OF NERVE GROWTH FACTOR BY PC12 CELLS: QUANTITATIVE ULTRASTRUCTURAL AUTORADIOGRAPHY, Ruth Hogue-Angeletti, Anna Stieber and Nicholas Gonatas, University of Pennsylvania, Philadelphia, PA 19104

Endocytosis of [^{125}I]nerve growth factor(NGF) by PC12 cells was studied by ultrastructural quantitative autoradiography. NGF-naive cells, which grow in clusters and lack neurites, were incubated at 37° with [^{125}I]NGF for periods of up to 26 hours. Labeling of plasma membrane was observed at all times. After 2 hours, endocytosis of [^{125}I]NGF was detected, with low levels of breakdown of the [^{125}I]NGF. Grain density distributions showed significant labeling of lysosomes, while nuclei and rough endoplasmic reticulum showed the lowest levels of labeling. Significant labeling of vesicles of the smooth endoplasmic reticulum and of various cytoplasmic components was also observed.

Our findings indicate that [^{125}I]NGF undergoes endocytosis quite slowly. During the initial phase of the interaction between NGF and PC12 cells, plasma membrane moieties are constantly labeled, while lysosomes show progressively increasing uptake of [^{125}I]NGF. The pathway of endocytosis of [^{125}I]NGF included vesicles of the smooth endoplasmic reticulum, but the Golgi apparatus was not unequivocally labeled. These results will be compared to results in PC12 cells which have grown neurites under the influence of NGF.

0376 RECEPTOR-MEDIATED BINDING AND INTERNALIZATION OF VITELLOGENIN BY XENOPUS OOCYTES, Lee K. Opresko and H. Steven Wiley, Calif. Institute of Technology, Corona del Mar, CA 92625 and University of California, Irvine, CA 92717

Vitellogenin (VTG), the estrogen-induced yolk precursor protein of oviparous vertebrates, is specifically sequestered by oocytes by receptor mediated endocytosis. The rate of VTG uptake can be modulated by hormones such as steroids and gonadotropins as well as long term exposure to VTG itself. We investigated several aspects of the binding and internalization of VTG using [^{32}P]VTG labeled in vitro by the action of a Xenopus protein kinase (Cell,22,47-57, 1981). At 0° VTG binding was saturable, reached equilibrium by 4 hr and was >98% specific at a concentration of 1 mg/mL (2.2 nM). Scatchard plot analysis of oocytes maintained at 0° revealed an apparent single class of binding sites whose number varied from 2.1×10^{10} - 1.4×10^{11}/oocyte depending on the donor animal. Preincubating oocytes with 3 mg/mL of VTG for 12 hr at 20° did not alter the number of cell surface receptors indicating that the VTG receptor is not "down regulated". Based on the rate of VTG uptake at 20°, oocytes internalize their total complement of receptors in a minimum of 7 - 24 min. Additionally, the total number of VTG surface receptors was found to be inversely proportional to the turnover rate of the oocyte surface. Our data is consistent with a model in which oocytes can modulate ligand uptake not only by the rate of receptor insertion into the cell membrane, but also by altering the number of receptor internalization sites at the cell surface.

0377 A STEADY STATE ANALYSIS OF EGF BINDING, INTERNALIZATION AND DEGRADATION: THE RELATIONSHIP BETWEEN RECEPTOR OCCUPANCY AND THE MITOGENIC RESPONSE, H. Steven Wiley, Daniel J. Knauer and Dennis D. Cunningham, University of California, Irvine, CA 92717

We have described a steady state model for analyzing the cellular binding, internalization and degradation of polypeptide ligands[1]. This model enabled us to describe the interaction of epidermal growth factor (EGF) with human and mouse fibroblasts as a set of rate constants. The rate constants were then utilized together with a computer simulation of the steady state model to find the relationships between the various cellular processes involved in hormone processing and the mitogenic response. We found that while human and mouse fibroblasts differed quantitatively with respect to their rate constants, their qualitative interaction with EGF was identical. In both cell types, "down regulation" of the EGF-receptor complex significantly increases the cellular affinity for the hormone (>10-fold). At steady state, ~90% of the EGF is associated with several intracellular compartments. Additionally, we found that there is a linear relationship between steady state receptor occupancy and the mitogenic response and that the mitogenic response is maximum when all cellular receptors are occupied at steady state. This indicates that the EGF-receptor complex is directly involved in the generation of the mitogenic signal and that there is only one functional class of EGF receptors on fibroblasts.

1. Cell 25:433-440 (1981).

0378 HUMAN GRANULOCYTES INTERNALIZE RADIOLABELED CHEMOTACTIC PEPTIDE INTO A GALACTOSYL TRANSFERASE RICH FRACTION CONTAINING THE LABEL IN AN APPARENT HIGH AFFINITY HIGH MOLECULAR WEIGHT FORM, Algirdas J. Jesaitis, Richard G. Painter, Larry A. Sklar, Manfred Schmitt, and Charles G. Cochrane, Scripps Clinic and Research Foundation, La Jolla, CA 92037

Neutrophils were exposed to 50 nM ^{3}H N-f-met-leu-phe (^{3}H-FMLP) for 1,5,10, and 25 min at 37°, washed, lysed by N_2 cavitation, and fractionated by isopycnic sucrose density gradient centrifugation. At one minute radioactivity was found in the plasma membrane and cytosol fraction. After 5,10, and 25 minutes radioactivity accumulated in the cytosol at a linear rate of 4000 molecules/cell-min but saturated in a low density (ρ=1.11) galactosyl transferase fraction at approximately 10,000 mol/cell. When cells were incubated with ^{3}H-FMLP at 4°C or in the presence of 10 fold excess nonradioactive FMLP, incorporation of radioactivity in both fractions was absent or 80 percent reduced respectively. Addition of excess nonradioactive FMLP after incubation of cells for 5 min with ^{3}H-FMLP caused a decrease in the amount of radioactivity in the galactosyl transferase fraction at 10 and 15 min. The radiolabel in the galactosyl transferase fraction remained 90 percent sedimentable inspite of dilution and multiple cycles of freeze thawing or sonication. Solubilization in Triton X-100 and passage over a Sepharose 4B column revealed that the radiolabel eluted in the void volume of the column well before elution of radiolabelled receptor from resting membranes. We conclude that neutrophils internalize chemotactic peptide rapidly and process it through a subcellular fraction which has a Golgi enzyme activity associated with it and that the peptide or a derivative is incorporated into a high molecular weight complex which dissociates very slowly.

0379 PHOTOAFFINITY LABELING OF THE FORMYLATED CHEMOTACTIC PEPTIDE RECEPTOR OF INTACT, FUNCTIONALLY COMPETENT HUMAN POLYMORPHONUCLEAR LEUKOCYTES (PMNL), Richard G. Painter Manfred Schmitt, Algirdas Jesaitis, Larry Sklar, Klaus Preissner, and Charles Cochrane, Scripps Clinic and Research Foundation, La Jolla, CA 92037

Quantitative analysis of ligand-occupied receptor interactions with elements of the cytoskeleton and with intracellular compartments requires a sensitive and simple method of identifying the receptor-ligand complex in living cells. Toward this goal, we have prepared a photoactivatable arylazide derivative of the chemotactic peptide, N-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys which can be radiolabelled to high specific activity with ^{125}I. This derivative was biologically active as judged by its ability to elicit superoxide anion production by human PMNL at nanomolar concentrations (ED_{50} ~ 0.8 nM). When incubated at 0° with whole PMNL, radioactive ligand became specifically and saturably associated with a 50-60K dalton species (as assessed by SDS-PAGE) in a light-dependent fashion. Addition of 50-100 fold excess of unlabelled parent or unlabelled azidopeptide derivative completely blocked uptake into this species. Approximately 15-20 percent of the available surface receptor binding sites were covalently labelled under these conditions. After labeling and irradiation the PMNL were fully capable of producing superoxide when challenged with various stimuli. Subcellular fractionation of the labelled cells on sucrose gradients after homogenization showed that the labelled species was primarily associated with plasma membrane-rich fractions. Stimulation with unlabelled peptide at 37° chased the label into fractions associated with low density galactosyl transferase-containing and granule-containing fractions.

0380 THE INTERNALIZATION OF N-FORMYL PEPTIDES BY NEUTROPHILS IS TOO SLOW TO BE INVOLVED IN RAPID RESPONSES INCLUDING FREE RADICAL PRODUCTION, SECRETION, AND MEMBRANE DEPOLARIZATION, Larry A. Sklar, Algirdas, J. Jesaitis, Richard G. Painter, and Charles G. Cochrane, Scripps Clinic and Research Foundation, La Jolla, CA 92037

We have compared the kinetics of the responses of neutrophils to the kinetics of ligand-receptor interaction and internalization, using as a model ligand the fluoresceinated hexa-peptide, N-CHO-nle-leu-phe-nle-tyr-lys-Fluorescein (nle, norleucine). The responses are all initiated within 10 seconds of the exposure of cells to stimulus. In the cases of membrane depolarization and secretion (in cytochalasin B treated cells), responses are elicited by binding which occurs within 15 seconds of peptide addition. Ligand binding and internalization have been analyzed over the same time frame with spectroscopic techniques. The association of ligand and receptor is monitored using an antibody to fluorescein. The antibody to fluorescein specifically quenches the ligand which is in solution, but receptor-bound ligand is inaccessible to the antibody. For an optimal dose of stimulus (i.e. ~ 1 nM), ~10-20 percent of the receptors (i.e. 10,000 of 50,000) are occupied in 15 seconds and contribute to the cell responses. The internalization of the receptor-bound ligand is monitored by the accessibility of the fluoresceinated peptide to quenching by an external pH change (7.4→2.5). Ligand which is either outside or on the cell surface is instantaneously quenched while intracellular peptide (or intracellular fluorescein derived from fluorescein diacetate) is only slowly quenched. No internalization is observed until 1 minute after binding begins and internalization proceeds at a rate of up to 5,000/min while the occupied receptors are being cleared from the surface.

0381 BINDING OF ^{125}I-ANGIOTENSIN II TO THE CELLS OF THE ADRENAL. AN ULTRASTRUCTURAL RADIOAUTOGRAPHIC STUDY. M. Cantin, S. Benchimol and J. Genest, Clinical Research Institute of Montréal, Montréal, Québec, Canada. H2W 1R7.

A single injection of ^{125}I-Angiotensin II (10^8 CPM; 40 ng) to nembutal-anesthetized rats (female, Sprague-Dawley; body weight: 40g) through an intraaortic catheter placed in the left carotid artery was followed 2 min. later by intracardiac perfusion of Ringer-Locke fluid for 30 sec. followed by 2% glutaraldehyde buffered with cacodylate-HCl (0.1 M pH 7.2) for 10 min. Fragments of cortex and medulla were left for 2 hrs in the same fixative, washed in cacodylate buffer containing 2% sucrose and post fixed in 2% osmium tetroxide buffered with Veronal acetate. The fragments were then processed for ultrastructural radioautography using Ilford L4 emulsion and the flat substrate technique. After 2 months of exposure, most of the silver grains were found associated with the cells of the zona glomerulosa and of the adrenal medulla while a negligible part of the grains was found associated with the cells of the zona fasciculata and reticularis which thus served as an internal control. In the zona glomerulosa and in the adrenal medulla, most of the silver grains were found in close association with the plasmalemma of hormone-secreting cells. Parallel experiments indicated that at this time interval 31% of the radioactivity found after perfusion for 10 min. with glutaraldehyde (11,472 CPM) was lost during tissue processing, most of the loss occurring during the immersion stage of glutaraldehyde fixation. This is the first ultrastructural visualization of AII binding in vivo to the plasmalemma of zona glomerulosa cells. This study also confirms the presence of receptors for AII on the chromaffin cells of the medulla. (Supported by a MRC Group Grant).

0382 NUCLEAR ACTIONS OF INTERNALIZED EPIDERMAL GROWTH FACTOR IN RAT PITUITARY CELLS, Lorin K. Johnson and Norman L. Eberhardt, Stanford University, Stanford, Ca. 94305

Epidermal growth factor (EGF) is not mitogenic for GH_3cells but does induce cell elongation, inhibits thyroid hormone (T_3) stimulated cell growth, T_3 induction of growth hormone (GH) synthesis and stimulates prolactin (Prl) synthesis. EGF does not alter the response of GH and Prl to glucocorticoid treatment (Johnson et al, PNAS, 77, 1980). EGF produces a maximal inhibition of T_3 stimulated growth at 0.25ng/ml but is required at 25ng/ml to produce a maximal inhibition of T_3 stimulate GH synthesis, indicating that the mechanistic pathways for EGF regulation of cell growth and GH production may be dissociable. Because T_3 regulates the GH gene via an activation of GH transcription we have examined possible nuclear actions of EGF. Previous studies showed that immunologically intact EGF accumulates in GH nuclei and that this can be increased by inhibiting EGF degradation with chloroquine (Johnson et al, Nature, 287, 1980). The nuclear sites of EGF binding are high affinity (Kd=1 nM) and are not released by micrococcal nuclease digestion, implicating the innernuclear membrane or nuclear matrix fraction. SDS gels of the ^{125}I-EGF loaded nuclei revealed bands at 6K and 185K daltons the reported mol. wt. for the EGF-receptor complex in other cell-types. Examination of nuclear proteins on 2-D gels showed basic proteins (pI>8) at 30K and acidic proteins (pI 5.5) at 20K whose phosphorylation was regulated by EGF. Phosphorylation of the 20K proteins was induced by T_3 and glucocorticoids but was inhibited by EGF and to a greater extent by EGF in the presence of chloroquine. These phosphoproteins were not released from nuclei by DNAse I digestion or 0.4M KCL extraction. Intact, internalized EGF, may therefore directly influence nuclear phosphorylation reactions, which subsequently participate in altering gene activity.

0383 DIRECT STIMULATION BY INSULIN OF NUCLEOSIDE TRIPHOSPHATASE ACTIVITY IN PURIFIED NUCLEAR ENVELOPES, F. Purrello, R. Vigneri, G.A. Clawson, and I.D. Goldfine, Cell Biology Laboratory, Mt. Zion Hospital and Medical Center, and Departments of Medicine, Physiology and Pathology, University of California, San Francisco CA 94120

The nuclear envelope contains a unique enzyme, the nuclear envelope nucleoside triphosphatase (NTPase), that provides the energy to transport mRNA out of the nucleus. In light of the observation that insulin raises mRNA levels in various tissues and in view of the recent report that the direct addition of insulin to isolated nuclei markedly increases mRNA efflux, we investigated whether there is an effect of insulin on the NTPase activity of the nuclear envelope. Isolated nuclei were first prepared from the liver of diabetic rats, and then highly purified nuclear envelopes were isolated by the method of Monneron. The direct addition of insulin to the envelopes stimulated NTPase activity; at 10 pM, the concentration at which the insulin effect was maximal, enzyme activity was increased by 27.1 ± 1.1% (mean ± SE; n=10). The insulin analogs, desdipeptide proinsulin and desoctapeptide insulin, also stimulated enzyme activity in proportion to their reduced biological potencies. In contrast neither boiled insulin nor insulin B chain, Zn^{++}, growth hormone and CCK had any effect on enzyme activity. The major effect of insulin was to increase the Vmax of the NTPase; no significant effects were seen on Km. In contrast, under identical conditions, insulin had no effect on either Mg^{++} or Na^{+}-K^{+} ATPase activities in purified plasma membranes. The present study demonstrates therefore that insulin directly stimulates nuclear envelope NTPase. Furthermore these studies raise the possibility that one mechanism whereby insulin regulates mRNA levels is by increasing the transport of mRNA from the nucleus.

0384 POSSIBLE BIOLOGICAL ROLE OF THE NUCLEAR INSULIN RECEPTORS, Dorothy E. Schumm, The Ohio State University, Columbus, Ohio, 43210.

The addition of $3x10^{-7}$ M insulin to a cell-free RNA transport system causes a 50% increase in the amount of messenger-like RNA released from rat liver nuclei. Insulin concentrations in excess of $1.2x10^{-6}$ M are inhibitory. Treatment of nuclei with Triton X-100 to remove the outer nuclear membrane abolishes the insulin effect. Increasing amounts of cytosol protein causes an increase in the amount of insulin necessary for maximal stimulation. The same percent increase in RNA release occurs with incubations carried out at 18,25 and 30°C. This indicates that the insulin effect is primarily on mRNA transport, the limiting factor at these temperatures, rather than on processing. Nuclei prepared from the insulin-resistant Zucker rat (fa/fa) give no response to insulin while those from the lean heterozygous Zucker rat show an intermediate amount of response with the same optimal insulin concentration. Thus the defect in these animals may be the number of receptors rather than their affinity for insulin.

0385 ENDOCYTOSIS AND UNCOATING OF VESICULAR STOMATITIS VIRUS, Robert W. Van Holten, Douglas K. Miller, and John Lenard, CMDNJ-Rutgers Medical School, Piscataway, N.J.

Previous studies have suggested that vesicular stomatitis virus (VSV) enters cells by endocytosis and uncoats in an intracellular acidic milieu. Infection of BHK cells by VSV observed by *in vivo* RNA transcription can be reversibly inhibited by a variety of amines (such as methylamine) known to accumulate in and raise the pH of lysosomes and other acidic compartments (see Miller and Lenard, PNAS, 78, 3605, 1981). We have developed a subcellular fractionation scheme using one Percoll density gradient that successfully separates from each other lysosomes, mitochondria, free virus, light cellular membranes, and cytosol. This procedure has been used to follow the internalization of virus into cells after a synchronized infection (p.i.). Within 15 min ^{35}S-met labeled virus is partially endocytosed (becomes trypsin insensitive) and appears in regions of the gradient corresponding to light membrane vesicles and lysosomes. The amount of virus in the lysosomal pool peaks by 60 min p.i. Simultaneously with the appearance of virus in these intracellular organelles, a substantial portion of viral components appears as free TCA-insoluble material in the cytoplasm. SDS gels of this soluble material reveals the presence of much N (nucleocapsid) protein, little M (membrane matrix) protein, and no G (spike) glycoprotein. In similar incubations of cells with VSV in the presence of methylamine, this soluble viral material is not formed. When methylamine is washed out, the soluble viral material is again formed. These results suggest that uncoated virus is released into the cytosol. Parallel experiments with horseradish peroxidase show a similar rate of endocytosis with maximum incorporation into the lysosomal pool by 90 min after endocytosis. (Supported by NIH AI 13002 and CMDNJ MSRP 27-9618.)

0386 A NOVEL ENDOCYTIC PATHWAY INVOLVING "GOLGI" FOR EPIDERMAL GROWTH FACTOR PROCESSING, W. Keith Miskimins and Nobuyoshi Shimizu, Dept. Cell. and Dev. Biol., Univ. of Ariz., Tucson, AZ 85721

The endocytic pathway involved in the intracellular processing of EGF was studied using Percoll gradients. In the presence of serum EGF was initially taken up into a cellular component which had a density identical to that of Golgi markers. It was then translocated into a fraction which comigrated with lysosomal markers. A portion of the EGF remained, undegraded, in this fraction for up to 7 h. Further fractionation of this peak demonstrated that a majority of the subcellular structures containing EGF are separable from lysosomes. A much different pathway was observed in the absence of serum. The majority of internalized EGF became associated with a subcellular fraction which does not correspond to any of the markers for which we have tested. It is then translocated to the fraction which corresponds to lysosomes. In the absence of serum nearly all of the EGF in this fraction is degraded and released from the cells. In addition, we have observed differences in the binding and degradation in the presence or absence of serum. In the presence of serum, after 1-2 h at 37°C, binding reaches a maximum level which is then maintained for at least 6 more h. In serum free medium, however, binding reaches a maximum near 1 h and then declines over the next 6 h. The proportion of cell bound EGF which becomes degraded is decreased in the presence of serum. Thus, EGF appears to be processed through 2 separate pathways which may serve different functions and are regulated by serum factors or the physiological state in which they maintain cells. (Supported by grants from NIH, GM24375 and ACS, JFRA-9)

0387 SUBCELLULAR LOCALIZATION OF NERVE GROWTH FACTOR IN CULTURED SYMPATHETIC NEURONS. Philippa Claude, Edward Hawrot*, Robert B. Campenot+ and Isabel Parada. Reg. Primate Res. Ctr., Univ. of Wisconsin-Madison, *Dept. of Pharmacology, Yale Sch. of Med., New Haven, CN. and +Sect. Neurobiology and Behavior, Cornell Univ., Ithaca, NY.

Biologically active iodinated Nerve Growth Factor (^{125}I-NGF) was prepared using the 2.5S species from male mouse salivary glands. After exposing the distal neurites of rat sympathetic neurons to 50 ng/ml ^{125}I-NGF in the side chambers of compartmentalized culture dishes, we localized the NGF using EM autoradiography. Most of the label in the cell bodies was concentrated in lysosomes and multivesicular bodies (MVB). Lysosomes accounted for the largest fraction (45-60%) of the grains in the cell bodies, with a labeling density (LD = %grains/%area) of 3-5. MVB accounted for 5-10% of the grains, with a LD of 5-20. Even after 24 hours exposure to NGF, we observed no significant nuclear labeling (LD<0.2). Interestingly, the lysosomes in the proximal neurites were about twice as radioactive as those in the cell bodies, while the MVB were less than half as radioactive as those in the cell bodies.

In the presence of the lysosomal blocking agents methylamine (MA, 10 mM) and chloroquine (CQ, 0.05 mM) lysosomes appeared swollen with membranous material. However, the neurons still internalized ^{125}I-NGF. In MA the fractional area of MVB was markedly increased, but they were not heavily labeled. In CQ MVB were virtually absent and the lysosomes were highly labeled (LD=6). We observed no evidence for nuclear accumulation of NGF even in the presence of these lysosomtropic agents. (Supported by the MDA, the Helen Hay Whitney Foundation, the NSF, the NIH and the Base Operating Grant of the WRPRC).

0388 RECEPTOR-MEDIATED ENDOCYTOSIS OF HUMAN TRANSFERRIN AND ITS CELL SURFACE RECEPTOR. J.W. Larrick*, C.A. Enns*, H.A. Suomalainen+, J. Schroder+, H.H. Sussman*
*Stanford Med. CTR., Palo Alto, CA; +Folkhalsan Inst. Genetics, Helsinki, Finland.

The incorporation of iron into human cells involves the binding of diferric transferrin to a specific cell surface receptor. We have studied the process of endocytosis in K562 (myeloid) and CEM (T cell) cells using rhodamine (RD) or fluorescein (FL) labelled iron transferrin (Fe-TF) and FL-labelled F(ab) fragments of specific goat or mouse monoclonal antireceptor antibody. Because antireceptor antibody (TFR-AB) and Fe-TF bind to different sites on the TF receptor molecule, it is possible to simultaneously and independently follow receptor and ligand.

At 4°C binding of RD-FeTF or FL-anti-TFR-AB exhibit diffuse membrane staining. Binding of RD-FeTF causes rapid formation of surface aggregates at 20°C. A large aggregate of receptor and FeTF appears in the cytoplasm within several minutes of elevating cellular temp. to 37°C. Although the diffuse surface staining and aggregates are removed by proteolysis, the large intracellular aggregate is not susceptible to enzyme. The precise nature of this 'FeTF depot' is still under investigation.

Biochemical studies suggest that the 94K M_r TFR may form dimers in the membrane (JBC 256: 9820). Because transferrin consists of two highly homologous domains it is possible that endocytosis is triggered by 'crosslinking' of two disulfide-linked receptor molecules. The fact that RD-FeTF is rapidly endocytosed whereas Fl-F(ab) fragments of anti-TFR bind diffusely and are only slowly endocytosed is consistent with this model.

0389 ENZYME MODIFICATION OF EPIDERMAL GROWTH FACTOR RECEPTOR IN A431 PLASMA MEMBRANE PREPARATIONS, Robin W. Yeaton, Michael T. Lipari and C. Fred Fox, University of California, Los Angeles, CA 90024

Membrane preparations from A431 human epidermoid carcinoma cells contain a 160/145Kd, EGF-binding set of proteins as visualized by gel electrophoresis/autoradiography of protein bands direct labeled with ^{125}I-EGF (Linsley and Fox, Nature 278:745, 1979). In membrane preparations containing predominantly the 160Kd component, digestion with endogenous A431 protease converts the 160Kd direct labeled protein to 145Kd with no change in the total amount of direct labeled ^{125}I-EGF:EGF receptor complex. Direct labeled receptors in membrane vesicles, (1) solubilized in nonionic detergent or (2) treated by hypotonic shock to allow inwards transport of macromolecules, have greatly increased sensitivity to digestion by this endogenous enzyme or trypsin. In contrast, direct labeled receptors in vesicles not subjected to these conditions are relatively refractory to protease digestion. This provides evidence for sidedness in membrane vesicles of a protease-sensitive domain on EGF receptors. This research was supported by grants from USPHS, ACS and MDAA.

0390 EPIDERMAL GROWTH FACTOR IS NOT DEGRADED IN LYSOSOMES, B. Magun and J. Sorrentino, Anatomy Dept, Col. Med., Univ. Arizona, Tucson, AZ 85724

The intracellular fate of endocytosed ^{125}I-epidermal growth factor (EGF) was examined in rat fibroblasts, 3T3, NRK and A431 cells. Cells were incubated at 37° in serum-free medium containing 1 mg/ml BSA and ^{125}I-EGF for 30 min at which time the dishes were rinsed thoroughly and incubated at 37° for up to 5h in the same medium minus EGF. At times after removal of the ^{125}I-EGF, cells were harvested and the postnuclear supernatant was analyzed by isopycnic centrifugation on Percoll gradients. At early times after ^{125}I-EGF removal from the cells, the ^{125}I activity appeared in an organellar peak which included all the activity for acid phosphatase and other lysosomal enzyme markers. Shortly thereafter the ^{125}I activity disappeared from the acid phosphatase peak and instead appeared in a peak which remained on top of the gradient. Two h after removal of the ^{125}I-EGF from the cells ^{125}I activity began to appear in the culture medium. Analysis of the ^{125}I material in these fractions revealed that in both gradient peaks the ^{125}I activity was present in a high MW form which co-chromatographed with intact EGF, reacted immunologically with anti-EGF antibody, and rebound to fresh cells at least as well as intact ^{125}I-EGF. No degradation products were detected in either peak. The ^{125}I material which appeared in the medium at later times consisted of degraded ^{125}I material. From these data we conclude that ^{125}I-EGF is not degraded in lysosomes, but instead enters another compartment perhaps a non-organellar one, in an undegraded form. Transit from this second compartment to the cell exterior is coupled with degradation. Inhibition of mitogenic stimulation by lysosomotropic amines suggests that the EGF may act at cellular sites distal to incorporation into lysosomes.

0391 ENERGY INDEPENDENT DEGRADATION (INTERNALIZATION) OF ^{125}I-β NERVE GROWTH FACTOR, Robert W. Stach, S.U.N.Y. Upstate Medical Center, Syracuse, NY 13210

Nerve growth factor (NGF) is a polypeptide hormone that is necessary for the growth and development of the sympathetic and sensory nervous systems. It has been shown that NGF binds to its responsive cells through cell surface membrane receptors. Recently it has been shown that after NGF binds to the higher affinity, type I, receptor, a time and energy dependent process occurs that makes the NGF inaccessible to the external milieu (Olender & Stach, J. Biol. Chem. 255, 9338:1980; Olender, et al., J. Neurochem. 37, 436:1981). This inaccessible NGF has been termed sequestered NGF. It has also been shown that NGF could be degraded after binding to sensory nerve cells (Sutter, et al., J. Biol. Chem. 254, 5972:1979). We were interested in determining if sequestration was a necessary prerequisite for degradation of NGF by sensory nerve cells. Dorsal root sensory ganglia from 9 day embryos (E9) were dissociated and used in this study. The dissociated cells were incubated with 7.5 x 10^{-12} M ^{125}I-βNGF at 22° or 4° C for 30 to 45 minutes. After this time samples were brought to 37° C and incubated for an additional hour. Under these conditions, ∿17% of the NGF that was bound was degraded. Degradation does not occur at 4° C; or in the presence of NaF and DNP when added at the beginning of the experiment; or when excess βNGF is added from the beginning of the experiment. Degradation does occur if cells are incubated at 4° C, then the temperature raised to 37° with concomitant addition of excess βNGF; or if cells are incubated at 4°, then the temperature raised to 37° with the concomitant addition of NaF and DNP. This suggests that the sequestration of NGF is necessary for degradation to occur, but degradation (internalization) is energy independent. Kinetics of binding at 4° was also studied. Supported by NIH grant NS 12325.

0392 INSULIN INTERNALIZATION BY TRANSFORMED AND UNTRANSFORMED CELLS STUDIED BY FLOW CYTOMETRY, Robert F. Murphy, R. Scott Powers, Charles R. Cantor and Robert Pollack, Departments of Chemistry and Biology, Columbia University, New York, NY 10027

We have developed flow cytometric methods for measuring the kinetics of binding, internalization, and lysosomal entry of fluorescent derivatives of insulin in mouse cultured cell lines. Using these methods, Swiss 3T3 cells, which require insulin when grown in serum-free medium, were found to internalize labeled insulin with a saturable, first order dependence on insulin concentration. In contrast, SV101 cells, which do not require insulin under the same conditions, internalize less labeled insulin than 3T3 cells, and the internalization is linearly related to insulin concentration. AY4 cells, which are derived from SV101 but require insulin to grow, have insulin internalization properties similar to those of 3T3. 3T6 cells, which are not transformed but do not require insulin, internalize insulin in a manner similar to SV101. For these four cell lines, the amount of labeled insulin internalized shows a very high degree of correlation with the growth rate without insulin relative to that with insulin. This correlation suggests that insulin internalization is important in growth regulation. This work was supported in part by post-doctoral fellowship DRG-352-F from the Damon Runyon-Walter Winchell Cancer Foundation (R.F.M.), by NIH Grants GM-14825 and GM-27576 (C.R.C.), and by NCI Grant CA-25066 (R.P.).

0393 BIOLOGICAL MODIFICATION OF LDL INDUCES RECOGNITION BY MACROPHAGE RECEPTORS AND ENHANCES CHOLESTEROL ESTER ACCUMULATION, Eileen M. Mahoney, Tore Henriksen and Daniel Steinberg, University of California, San Diego, La Jolla, CA 92093

In vivo, macrophages accumulate large stores of cholesterol esters in their cytoplasm under certain pathological conditions. Yet, in vitro, macrophages do not avidly metabolize LDL, the major plasma carrier of cholesterol. We report that incubation of LDL with endothelial cells converts LDL from a form that is slowly internalized and degraded by macrophages to one that macrophages recognize specifically and internalize 3-4 times more rapidly. Degradation of the endothelial cell-modified LDL displays saturation kinetics and competes for degradation of chemically acetylated LDL, for which receptor sites have previously been demonstrated uniquely on macrophage surfaces. Metabolism of endothelial cell-modified LDL leads to increased acyl coA : cholesterol acyltransferase activity with the result that the cholesterol liberated from the endothelial cell modified LDL during degradation is esterified in the cytoplasm and accumulates throughout the incubation period. However, unlike native LDL matabolism in fibroblasts, the rate of macrophage degradation of endothelial cell-modified LDL remains constant for several days in culture and evidently does not lead to regulation of the surface receptor. Therefore, interaction between LDL and endothelial cells generates a modification of the lipoprotein that permits recognition of it by macrophage surface receptors with subsequent intracellular degradation of the LDL and cholesterol ester accumulation.

0394 NUCLEAR RECEPTORS FOR GONADOTROPINS AND PROSTAGLANDINS. Ch. V. Rao and S.B. Mitra, Depts. of Ob/Gyn & Biochem., Univ. of Louisville, Louisville, KY 40292

Nuclei (N) isolated from bovine corpora lutea (bCL) were enriched with NAD pyrophosphorylase but contained very little or none of other marker enzymes. These N specifically bound added ^{125}I-human chorionic gonadotropin (^{125}I-hCG) and ^{3}H-prostaglandin (PG) E_1 and $F_{2\alpha}$. However, these N bindings were only 8-15% of bindings observed for plasma membranes (PM) isolated from the same bCLs. Nuclear membranes (NM) that were free from chromatin as assessed by the lack of NAD pyrophosphorylase, bound all three labeled ligands to at least the same level as the N. Chromatin that was free of NM as assessed by the lack of NADH cytochrome c reductase, also bound these labeled ligands. However, these bindings were lower than for N or NM. The ^{125}I-hCG specific binding to NM was heterogeneous (Kds-11&23x10^{-11}M) as compared to homogeneous (Kd-8.6x10^{-11}M) binding to PM. Numerous differences as well as similarities were found between NM & PM gonadotropin receptor properties. Luteal N and PM gonadotropin receptors increase from early to mid and then decrease in late luteal phase, paralleling the corpus luteum secretory activity. The ^{125}I-hCG internalized in bCL slices and was found to be associated with N and the other intracellular organelles which contain intrinsic gonadotropin receptors. The N had less ^{125}I-hCG uptake than any other intracellular organelle. Eluted N radioactivity was able to rebind to fresh PM but to a lesser extent than fresh ^{125}I-hCG. It came off Sepharose 6B columns with the same Kav as native ^{125}I-hCG. Detergent solubilized N radioactivity eluted as a macromolecular bound complex from the same columns (Support-NIH HD 14697).

0395 RECEPTOR AND NON-RECEPTOR MEDIATED UPTAKE AND DEGRADATION OF INSULIN BY HEPATOCYTES, David B. Donner, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

Physiologic and supraphysiologic amounts of insulin are degraded intracellularly by rat hepatocytes. Native insulin inhibits the binding and degradation of ^{125}I-insulin in parallel. Half-maximal inhibition of degradation occurs in the presence of 10 nM insulin, a hormone concentration at which the physiologically-functional insulin receptor is saturated. At 4^{o}C or 37^{o}C the proportion of degraded hormone increases as insulin interacts with low affinity sites. Thus, all bound insulin, whether receptor associated or not, is a potential substrate for degradation. In the presence of 0.6 nM insulin, a concentration at which most cell-associated hormone is receptor bound, chloroquine increases the amount of ^{125}I-insulin retained by hepatocytes. However, chloroquine increases the retention of degradation products of insulin in incubates containing sufficient hormone to saturate the receptor and permit occupancy of low affinity sites. Glucagon (50 μM) does not compete for the interaction of ^{125}I-insulin (1 nM) with the insulin receptor, but does inhibit the uptake of insulin (100 nM) by low affinity sites. The inability of glucagon to compete for insulin binding to high affinity sites, while inhibiting low affinity uptake, precludes the possibility that receptor binding is an obligatory step preceeding degradation at high hormone concentrations. It is proposed that insulin degradation is mediated by receptor and non-receptor pathways. The latter may be related to the actions of glutathione insulin transhydrogenase since both insulin and glucagon interact with this enzyme. Supported by grants AM 22121 and CA 08748 from NIH.

Growth Factors and Cell Division: Receptor-Linked Systems

0396 COLONY STIMULATING FACTOR (CSF-1) UPTAKE AND DESTRUCTION BY MACROPHAGES
L.J. Guilbert, R.J. Tushinski and E.R. Stanley, Departments of Microbiology and Immunology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461.

Mononuclear phagocytic cells and some macrophage cell lines have a highly specific binding site for the colony stimulating factor subclass CSF-1. CSF-1 is a glycoprotein that specifically stimulates mononuclear phagocyte survival, proliferation and differentiation in culture (1). However, the carbohydrate is not required for high affinity cell surface binding or biological activity.

Quiescent CSF-1 responding cells may be obtained by culturing bone marrow derived macrophages (BMM) in the absence of CSF-1. Following addition of ^{125}I-CSF-1 to these cells, it is bound to a high affinity site and is rapidly internalized ($t_{\frac{1}{2}}$42s). All of the internalized ^{125}I-CSF-1 is destroyed, and the entire destruction can be described by simple first order kinetics ($t_{\frac{1}{2}}$16m). During this initial uptake of ^{125}I-CSF-1, cell surface binding sites rapidly vanish and on removal of ^{125}I-CSF-1 very slowly reappear ($t_{\frac{1}{2}}$>4h). However, cells cultured continuously in the presence of CSF-1 (proliferating BMM) maintain a much higher steady state level of receptor mediated CSF-1 uptake and destruction than would be predicted from the receptor numbers and receptor turnover rates of quiescent cells. [The number of CSF-1 molecules per cell destroyed in one hour by proliferating BMM is greater than the number of sites on a quiescent cell.] The CSF-1 stimulated CSF-1 receptor turnover may be an important aspect of the proliferative response of BMM to CSF-1.

1. E.R. Stanley et al. This meeting.

0397 REGULATION OF MACROPHAGE PROLIFERATION BY A COLONY STIMULATING FACTOR (CSF-1)
R.J. Tushinski, I.T. Oliver and E.R. Stanley, Departments of Microbiology & Immunology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461.

A colony stimulating factor (CSF-1) is required for the survival, proliferation and differentiation of macrophages (1). Studies on the regulation of macrophage proliferation by CSF-1 were carried out in homogeneous populations of bone marrow derived macrophages (2).

Removal of CSF-1 from exponentially growing cells resulted in: (i) a decrease in the incorporation of [^{3}H] TdR into nuclei [from 33% to <5% at 12 h and to <1% at 24 h]; (ii) a 2.5-fold greater rate of loss of [^{3}H] Leucine labeled protein than in cells remaining in the presence of CSF-1, and (iii) the reduction of protein synthesis to one half that of exponentially growing cells. The increase in the rate of apparent degradation of protein (ii) and the decrease in the protein synthetic rate (iii) accounts for the absence of protein accumulation in non-proliferating macrophages. The addition of CSF-1 to non-proliferating cells resulted in a relatively synchronous stimulation of DNA synthesis within 12 h and an increase in the net accumulation of protein within 2-4 h. Exposure of non-proliferating macrophages to CSF-1 for varying periods indicates that CSF-1 is required during the G1 phase for cells to enter S phase, but not for the passage of the cells through S phase. These data indicate that in serum-containing macrophage cultures, CSF-1 regulates protein turnover and is required for events occurring in G1 which are critical for the entry of cells into S phase.

1. E.R. Stanley et al. This meeting.
2. R.J. Tushinski et al. (1982) Cell (in press).

0398 IN VITRO RADIATION SENSITIVITY OF CSF-DEPENDENT MURINE HEMATOPOIETIC STEM CELLS AND STROMAL SUPPORTIVE CELLS DIFFERS SIGNIFICANTLY.
R.R. Weichselbaum, M.A. Sakakeeny, A. Schmidt, J.B. Little, and J.S. Greenberger. Joint Center for Radiation Therapy, Dept of Radiation Therapy, and Harvard School of Public Health, Boston, MA. 02115.

Purified murine hematopoietic stem cells were separated by removal of nonadherent cells for continuous mouse bone marrow cultures at day 40. The cells were tested for gamma irradiation sensitivity (140 KVP, GE Maximar) by colony formation (greater than 50 cells per colony) following transfer to 0.8% methylcellulose-containing medium in the presence of L-cell colony stimulating factor for hemopoietic cells (CSF). The Do was 90-100 rad for NZB or NIH Swiss marrow culture CSF-dependent colony-forming cells. In contrast, when fresh bone marrow was tested in the same assay, a curve was observed that had an initial Do of 90-100 and resistant tail of 100-250. Separation of adherent from nonadherent cells was achieved by transfer of fresh bone marrow to empty petri dishes in the absence of CSF. These conditions allowed attachment of adherent stromal cells that did not require CSF for growth and allowed analysis of the adherent cell population alone. Under these conditions, a Do of 120-140 was obtained. These data provide strong evidence that the hematopoietic stem cell compartment (Do 90-120) and bone marrow stromal cell compartment (Do 120-250) have inherently different radiobiologic properties. Studies of radiation effects on whole bone marrow must consider these differences.

0399 HUMAN INTERFERON: BINDING TO AND DEGRADATION BY A HUMAN CELL LINE, Andrew A. Branca and Corrado Baglioni, State University of New York at Albany, Albany, New York 12222

Binding of ^{125}I-labelled human interferon (^{125}I-Hu-IFN-αA) to specific receptors on Daudi cells was demonstrated by the ability of unlabelled Type I human interferons (Hu-IFN-α,Hu-IFN-β) to compete for the binding of ^{125}I-Hu-IFN-αA. The association of the label with the cells was characterized by equilibrium and saturation binding kinetics. Competition binding experiments with a variety of Type I human interferon prparations indicated that these interferons bind to the same receptor with relatively equal affinities. Unlabelled Type II human interferon (Hu-IFN-γ) or mouse interferon (Mu-IFN-β) did not compete significantly for the binding of ^{125}I-Hu-IFN-αA indicating that Types I and II human interferons do not share the same receptor and reflecting the known species specificity of Mu-IFN-β.

The association of ^{125}I-IFN-αA with the cells exhibited a marked temperature dependence. Evidence from competition displacement experiments and characterization of the label released from the cells indicated the possible internalization of interferon and demonstrated directly its degradation by the cells. Correlations of label associated with preparations of cytoskeletal structures implicated a possible role for the cytoskeleton in the processing of interferon after initial binding to cell surface receptors.

0400 ECTOPIC PRODUCTION AND UTILIZATION OF GROWTH FACTORS BY CULTURED CELLS, Paul L. Kaplan and Brad Ozanne, Univ. of Texas Health Sci. Ctr., Dallas, TX 75235

Kirsten murine sarcoma virus (KiMSV) induces the production fo Transforming Growth Factors (TGFs), some of which compete with epidermal growth factor (EGF) for cell surface binding. KiMSV transformed fibroblasts grow in serum free medium devoid of exogenous mitogenic growth factors. We have examined cells of several species for their serum free growth requirements before and after transformation by KiMSV. The nontransformed cells all require the addition of at least EGF to the serùm free medium for growth, while the transformed cellsare EGF independent. Fürther, conditioned medium from the transformed cells obviates the EGF requirement of the nontransformed cells suggesting that KiMSV transformed cells are autocrine systems producing and utilizing EGF-like growth factors.

We also find that both EGF and TGF(s) induce normal rat and mouse cell lines to grow in soft agar, however the efficiency of growth induction varies with both the test cell and the growth factor. The interaction of different growth factors with the same receptor can lead to quantitatively different responses by the cell. This variation in sensitivity of different cells to both EGF and TGF(s) correlates with the focus forming ability of both KiMSV and Abelson murine leukemia virus and suggests that the EGF receptor is intimately involved with cell transformation.

0401 METHODS FOR RAPID MICROANALYSIS OF TRANSFORMING GROWTH FACTORS (TGFs) AND TUMOR CELL INHIBITORY FACTORS, Kenneth K. Iwata, Charlotte M. Fryling and George J. Todaro, Laboratory of Viral Carcinogenesis, NCI, Frederick, MD 21701

Transforming growth factors (TGFs) as well as inhibitors of tumor cell growth have been isolated in this laboratory from the supernatant fluids of human tumor cell cultures. An important element in any purification scheme for factors produced by tumor cells are assays which utilize a minimum amount of material and allow rapid, quantitative analysis of fractions from gel filtration, ion exchange chromatography, or high pressure liquid chromatography (HPLC). Modifications of assays using iodonitrotetrazolium and iodinated nucleotides for the rapid analysis of fractionated supernatant fluids from human tumor cell cultures have been developed. Assays are conducted in 96-well tissue culture clusters using 100 µl samples in triplicate incubated for 4 days. We have obtained good reproducibility in our replicate samples as well as between experiments. The results from the microassays are comparable with assays previously reported from this laboratory for testing stimulatory and inhibitory activities from cell supernatant fluids. Using the microassays, we observed different degrees of sensitivity to the TGFs and inhibitors of tumor cell growth by different tumor cell lines. Each tumor cell line may possess a characteristic responsiveness to TGFs and inhibitors of tumor cell growth.

0402 IN SITU MOLECULAR WEIGHT OF EPIDERMAL GROWTH FACTOR RECEPTOR, Harry T. Haigler, David End, and Ellis S. Kempner, Univ. of Calif., Irvine, CA 92717 and Natl. Inst. of Health, Bethesda, MD 20205.

We have used high energy radiation inactivation to determine the functional size of the epidermal growth factor (EGF) receptor in situ. The method is based on the random nature of the interaction between ionizing radiation and the target molecules and the complete loss of function of any polymer that is struck. Membranes isolated from the human epithelioid carcinoma cell line A-431 were exposed to increasing doses (0.5-72 Mrads) of high energy electrons and then assayed both for ^{125}I-EGF binding activity and for the activity of the added internal standard malic dehydrogenase. The log of the fraction of remaining activity was plotted as a function of the dose of radiation and from the resulting monoexponential decay, the target size was determined. The functional unit of malic dehydrogenase was found to be 70 kd and is in agreement with the known structure and radiation sensitivity of the enzyme. In several irradiation experiments, we obtained values for the ^{125}I-EGF binding component ranging from 130 kd to 170 kd, with the average being 154 $\pm$ 19 kd. The monoexponential nature of the decay indicates that only a single size of functional receptor exists. Target size analysis of the total number of binding sites determined from Scatchard plots gave a similar functional size (148 kd). Thus, the in situ molecular size of the EGF binding activity is in good agreement with the molecular weight of the EGF receptor obtained by SDS- PAGE of both the affinity-purified and the photoaffinity-labeled receptor.

Preliminary experiments suggest that the target size of the EGF-stimulated kinase activity in A-431 membranes is significantly smaller than the 150 kd EGF receptor.

0403 INTERACTION BETWEEN PLATELET-DERIVED GROWTH FACTOR AND ITS FIBROBLAST RECEPTOR, C.-H. Heldin, B. Ek, A. Wasteson and B. Westermark, University of Uppsala, Sweden.

A specific, high-affinity receptor for platelet-derived growth factor (PDGF) was recently demonstrated on connective tissue derived cells and glial cells (C.-H. Heldin, B. Westermark and A. Wasteson (1981) Proc. Natl. Acad. Sci. USA 78, 3664). We show here that the cell bound ^{125}I-PDGF is internalized and degraded; after one hour of incubation at 37°C, 90% of the cell bound radioactivity is recovered in a TCA-non-precipitable form in the cell culture medium. The degradation is impaired by inhibitors of lysosomal degradation such as chloroquine (50 μM), methylamine (10 mM) and ammonium chloride (10 mM), suggesting that the degradation occurs in the lysosomes. Binding of PDGF to its fibroblast receptor results in a concentration dependent decrease of cellular PDGF-binding capacity, i. e. down-regulation of the receptor. Reappearence of the receptor is dependent on protein synthesis; it was totally blocked by cycloheximide (20 μg/ml). This suggests that the receptor is degraded together with PDGF after internalization, or alternatively, that any step in the recycling of "used" receptors is dependent on protein synthesis. Homobifunctional (disuccinimidyl suberate and dithiobis(succinimidyl propionate)) or heterobifunctional (N-hydroxy succinimidyl 4-acido benzoate) cross-linkers have been used to stabilize the binding of ^{125}I-PDGF to its receptor. The ^{125}I-PDGF-receptor complex migrates as a 200 000 dalton component in SDS-gel electrophoresis performed under reducing as well as non-reducing conditions. This suggests that the receptor molecule is a single chain protein. Subtraction of the molecular weight of PDGF itself (30 000) yields a molecular wieght of about 170 000 for the PDGF-receptor on human fibroblasts.

0404 IDENTIFICATION OF A CELL SURFACE RECEPTOR FOR PLATELET-DERIVED GROWTH FACTOR (PDGF) BY AFFINITY LABELING, Kevin C. Glenn, BRL, P.O.Box 6009, Gaithersburg, MD 20877, Dan F. Bowen-Pope and Russell Ross, University of Washington, Seattle WA 98195.

Ethylene gylcol bis (succinimidyl succinate) and disuccinimidyl succinate, two homobifunctional crosslinking reagents, have been used to crosslink ^{125}I-PDGF to a protein with an approximate M_r=164,000. Increasing the concentration of unlabeled PDGF resulted in a parallel decrease in the amount of labeling of this component. However, high concentrations of epidermal growth factor, fibroblast growth factor, insulin, low density lipoprotein (LDL), or acetylated LDL had no effect. In addition, preincubation of cells with ^{125}I-PDGF at 37°C induced a reduction (downregulation) of specific ^{125}I-PDGF binding and a parallel decrease in the amount of the 164,000 dalton protein available for labeling. Thus, this protein has many characteristics expected of a specific receptor for PDGF. This receptor is a protein that is accessible to cleavage by trypsin in the extracellular medium. It appears that the receptor for PDGF is similar in size on four different cell types which are mitogenically responsive to PDGF (Swiss 3T3, WI-38, human and monkey arterial smooth muscle cells), but appears to be absent on cells that are not mitogenically responsive to PDGF (arterial endotehelial and A-431 cells). ^{125}I-PDGF does not become covalently crosslinked to this component in the absence of a crosslinking reagent or under conditions where a reversible reagent has been hydrolyzed subsequent to crosslinkage.

0405 A COMPARISON OF BOVINE BRAIN AND PITUITARY FIBROBLAST GROWTH FACTORS. Sandra K. Lemmon, Marian C. Riley and Ralph A. Bradshaw, Wash. U. School of Med. St. Louis MO 63110; Kenneth A. Thomas, Merck Research Laboratories, Rahway NJ 07065; Gail A. Hoover and Thomas Maciag, Harvard Med. School, Beth Israel Hospital, Boston MA 02215.
Bovine pituitary and brain fibroblast growth factors (FGFs)(1) were originally described as basic mitogens with a similar spectrum of activities. Subsequently, brain FGF was identified as fragments of myelin basic protein (MBP)(2), but this was disproven by Thomas et al (3) who showed that brain FGF activity is associated with an acidic molecule. A similar analysis (IEF) has confirmed that pituitary FGF is basic (pI 8-9), as reported (1). Brain FGF, but not pituitary, is mitogenic for human umbilical vein endothelial (HUVE) cells, an additional distinguishing property. However, comparison by acid gel electrophoresis of freshly prepared pituitary FGF with brain FGF stored for three years showed each contained a single activity that migrated to the same position. In contrast, more recently prepared samples of brain FGF showed, by IEF, the presence of the acidic component (pI 5-6) seen previously (3), and an activity indistinguishable in mobility from pituitary FGF. None of the activities comigrated with fragments of MBP. The aged brain FGF preparations were not active on HUVE cells and did not contain the acidic component. Thus, brain FGF contains two different mitogens which may occur in variable amounts. The acidic molecule apparently contains the HUVE cell activity and the basic component is similar to and may be identical with pituitary FGF.
1. Gospodarowicz, D. and Moran, J.S. (1976) Ann. Rev. Biochem. 45, 531-558.
2. Westall, F.C. et al (1978) Proc. Natl. Acad. Sci. USA 75, 4675-4678.
3. Thomas, K.A. et al (1980) J. Biol. Chem. 255, 5517-5520.

0406 PURIFICATION OF INTERLEUKIN-2 BY AFFINITY CHROMATOGRAPHY, Roberto Fagnani, John Mendelsohn, and Herbert L. Cooper, University of California, San Diego, La Jolla, CA 92093 and the National Cancer Institute, Bethesda, MD 20205
Interleukin-2 (IL-2) is a low molecular weight growth factor present in the culture supernatant from PHA-stimulated, human or murine lymphocytes (Conditioned Medium, CM). This factor enables the long-term "in vitro" proliferation of normal T cells, with full retention of their immunological functions. CM was produced by 24-hr, PHA-stimulated lymphocytes cultured in the absence of serum. A nearly complete one-step purification of IL-2 was achieved by affinity chromatography on Amicon Blue A gel. After adsorption, growth factor activity could be eluted with a linear salt gradient. PHA used to generate CM did not bind to the affinity gel: unstimulated lymphocytes, known to respond to PHA but not to IL-2, proliferated only when exposed to the run off from the column.

SDS-PAGE analysis of the purified IL-2 showed a contaminant in the 68,000 MW region, which was removed by filtration on a Sephadex G75 column. IL-2 molecules immobilized on Blue A gel beads retained growth-supporting capacity for human cultured T cells comparable to that induced by CM. This may indicate that internalization of IL-2 molecules is not a necessary event for the expression of the biological activity. The availability of radiolabelled IL-2 molecules will permit the characterization of the IL-2 receptor on activated T cells and on malignant lymphocytes, as well as a kinetic analysis of receptor expression following activation. Moreover, studies with pure IL-2 will determine the kinetics of receptor internalization and/or "down-regulation." Eventually, immobilized IL-2 molecules also will enable initial affinity purification of the receptor.

0407 THE EFFECT OF INTERLEUKIN-3 ON SELF-RENEWAL IN SUSPENSION OF MULTIPOTENTIAL HEMATOPOIETIC PROGENITOR CELLS. J.S. Greenberger, J. Ihle, G. Nabel, A. Hapel, and M.A. Sakakeeny. Joint Center for Radiation Therapy, Dept of Radiation Therapy, and Dept of Immunology, Sidney Farber Cancer Institute, Boston, MA. 02115, and Frederick Cancer Research Institute, Frederick, MD. 21701.

The methods for purification of Interleukin-3 (IL-3), a 41,000 MW glycoprotein found in lectin-treated spleen-conditioned medium, or in medium conditioned by the WEHI-3 cell line or other cell lines have been reported (Ihle, J., et.al., J. Immunol., 126:2184-2189, 1981). Purified preparations of IL-3 were tested for growth stimulation of cloned populations of a multipotential cell line called B6SUTA cl 5 cells. The B6SUTA cell line and its subclones have been passaged in WEHI-3 conditioned medium for over 2 years and produce hemoglobinized erythroid colonies (BFUe), as well as metachromasia-positive mast cell/basophil and neutrophilic granulocyte and macrophage colonies in vitro. Concentrations of 0.1 ng/ml of IL-3 stimulated growth of B6SUTA and subclonal cell lines. The cell lines absolutely required IL-3 for growth and died at a rate of one 10-fold dilution each 12 hours following removal from IL-3. These data provide evidence that a population of multipotential hematopoietic stem cells responds to a lymphokine growth factor that has been shown to stimulate 20-alpha-SDH synthesis by other lymphocyte populations.

0408 IDENTIFICATION OF GROWTH FACTORS IN HUMAN AND BOVINE CARTILAGE, Marjorie C. Bekoff and Michael Klagsbrun, Children's Hosp. Med. Center, Harvard Med. School, Boston, MA 02115.

Growth factor activity, as measured by the stimulation of DNA synthesis and cell division in cultured cells, can be detected in chondrocytes and extracellular matrix (ECM) obtained from bovine articular and scapular cartilage, and from human costal cartilage. The growth factor activity found within the chondrocyte is associated with the chromatin fraction of the cell. NaCl, at concentrations between 0.25M and 0.75M, will elute all of the mitogenic activity from chromatin. Chromatin-associated growth factor (CAGF) is not a histone and has a molecular weight (M.W.) between 20,000 and 22,000 as measured by gel filtration on HPLC TSK 3000 columns equilibrated with 6M guanidine HCL and 5mM dithiothreitol, pH 6.5. The factor found in the ECM, referred to as cartilage-matrix growth factor (CMGF), has a M.W. of 16,000-17,000. CAGF and CMGF adhere to the cation exchanger Biorex 70 and are eluted with 0.3-0.5M NaCl. The majority of non-histone chromosomal proteins are eluted from Biorex 70 with 0.1M NaCl, and the 5 histones require greater than 1M NaCl for elution. Both the cellular factor and the factor found in the ECM have similar properties such as being cationic and being resistant to inactivation by DTT and guanidine. It is possible that the CAGF with a M.W. of 20-22,000 is a precursor of the 16-17,000 M.W. growth factor found predominantly in the ECM. A monoclonal antibody to CMGF has been produced and is being used to localize the growth factors in cartilage and in cultured cells, and to study their structural relationship.

0409 ISOLATION AND PARTIAL CHARACTERIZATION OF TWO PORCINE PLATELET PROTEINS ASSOCIATED WITH MITOGENIC ACTIVITY FOR 3T3 CELLS, Andreina Poggi, Boguslaw Rucinski, Pranee James, John C. Holt and Stefan Niewiarowski, Thrombosis Ctr., Temple Univ. Health Sciences Ctr., Phila., Pa. 19140, USA, and Istituto Mario Negri, Milano 20157 Italy.

Human platelets are the source of potent mitogens for fibroblast-like cells: the Platelet Derived Growth Factors (PDGFs). Very little is known about the mitogenic activity of proteins secreted from platelets of mammalian non-primate species. We have isolated from porcine platelets two proteins associated with cell stimulating activity. Material released upon aggregation from washed porcine platelets was separated by affinity chromatography on heparin-agarose. Two fractions: crude PDGF, eluted at .6-.9 M NaCl and Platelet Basic Protein (PBP) eluted at .9 M NaCl were active as tested by ^{3}H-methyl-thymidine incorporation assay on 3T3 cells. TCA-precipitated radioactivity was measured and data expressed in units, one unit being the concentration of calf serum giving half maximal stimulation. The specific activity of the two proteins were .5 U/µg and .1 U/µg, respectively. Both proteins were further purified by gel filtration in 1 M acetic acid through a Sephacryl S200 column. Active PDGF appeared to be a protein of approximate molecular weight 25-30 K. Its specific activity was of 1.09 U/µg. PBP was eluted as a protein of 15-17 K; its specific activity was less than .1 U/µg. In conclusion, these data show that porcine platelets released two proteins associated with mitogenic activity for 3T3 cells. The one with higher activity showed many characteristics (molecular weight, affinity for heparin) in common with human PDGF.

0410 AN INTERFERON-SUSCEPTIBLE GROWTH FACTOR OF PLACENTAL ORIGIN, Patton T. Allen, Owen S. Weislow, Frank A. Snyder, and Arnold K. Fowler, NCI, FCRF, Frederick, MD 21701

Bioregulatory system components including interferons and growth factor receptors have been reported in placental tissues. We present here initial observations demonstrating the isolation of growth factor(s) from murine placental tissue, and a preliminary characterization of the factor(s). Aqueous tissue extracts of term placenta from the NIH Swiss mouse exhibited mitogenic activity in quiescent confluent cultures of a variety of cell lines derived from mouse and rat tissues. Anchorage independent growth of rat cells was also induced by the placental substances. In rate zonal sedimentation analysis at pH 7.4 the principal mitogenic activity co-sedimented with a 30,000 molecular weight (mol. wt.) reference marker. However, in gel exclusion chromatography at pH 3.5 the apparent mol. wt. of the placental factor was approximately 14,000 suggesting a subunit composition. The activity of the factor was moderately stable when exposed to thiol reagent, low pH, and 56°, but was rapidly destroyed at 80°, or by trypsin. By comparative analysis the placental growth factor was distinguishable from epidermal, fibroblast, platelet-derived, and nerve growth factors based on specific cell line response and/or heat and thiol reagent stability. Mitogenic response of murine cells to placental growth factor was blocked by 500 reference units/ml of murine interferon whether added 24 hrs. before the factor or simultaneously with the factor. For maximum inhibitory response the continued presence of interferon was required. We conclude that the placenta contains a soluble protein growth factor, distinct from the other factors chosen for comparison. A detailed characterization requires additional purification, and further comparative analysis.

0411 PROTEASE-NEXIN NEGATIVELY REGULATES THE INITIATION OF CELL DIVISION BY THROMBIN, David A. Low, Joffre B. Baker, Randy W. Scott and Dennis D. Cunningham, University of California, Irvine, CA 92717

Thrombin is a potent mitogen for a variety of cultured fibroblasts. We previously showed that cells release a protein that we named protease nexin, which links to thrombin and certain other serine proteases added to cell-conditioned medium. The resultant thrombin-protease nexin complexes bind to and are rapidly internalized and degraded by cells. To explore the physiological functions of protease nexin we have examined the effect that cell-released protease nexin has on the mitogenic stimulation of cells by thrombin. We find that conditioning of the medium by cells renders them less sensitive to stimulation by thrombin. Importantly, quantitation of the amount of thrombin and thrombin-protease nexin complexes present both in the media and associated with cells shows that the reduction of free thrombin in the medium resulting from linkage to PN can quantitatively account for the observed decrease in cellular sensitivity to thrombin. Finally, we find that a highly purified fraction of protease nexin (about 1000-fold) significantly shifts the thrombin dose response curve to less sensitive levels but does not significantly affect the response of cells to epidermal growth factor. Taken together, these results show that cells can specifically regulate their mitogenic response to thrombin by release of protease nexin.

0412 REGULATION OF THE EXTRACELLULAR EXPRESSION OF SECRETED PROTEINS IN 3T3 CELLS BY PEPTIDE GROWTH FACTORS AND LYSOSOMOTROPIC AGENTS, Richard T. Hamilton, Marit Nilsen-Hamilton and W. Ross Allen, Cell Biology Laboratory, The Salk Institute, San Diego, CA 92138

Peptide mitogens such as epidermal growth factor and fibroblast growth factor selectively increase the extracellular appearance of four proteins secreted by 3T3 cells. These proteins are, "mitogen regulated protein" (MRP) (Mr 34,000), "major secreted protein" (MEP) (Mr 37,000), and superinducible proteins I and II (SIPI and SIPII) (Mr 25,000 and 12,000). Our evidence suggests that the growth factors regulate the extracellular concentrations of these proteins at the level of transcription. Also, the extracellular concentrations of MRP and MEP, but not SIPI and SIPII, appear to be regulated by a mechanism involving degradation in the lysosomes. The amount of MRP and MEP is increased at least 2- to 3-fold by lysosomotropic agents such as ammonium chloride, methylamine and chloroquine, and by nigericin, and high extracellular pH. All of these agents decrease protein degradation in the lysosomes, probably by increasing the intralysosomal pH. Both MEP and MRP are glycoproteins although glycosylation is not necessary for their secretion. Monensin, nigericin and the lysosomotropic agents increase the extracellular concentrations of the glycosylated and not the nonglycosylated forms of both of these proteins. These results suggest that the glycosylated and nonglycosylated forms of MRP and MEP enter different compartments in the cell, and the compartment entered by the glycosylated forms is either the lysosomal compartment or a vesicle which has a probability of fusing with the lysosomes which then results in the degradation of MRP and MEP.

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0413 ASSOCIATION OF THE D-GLUCOSE TRANSPORTER WITH THE CYTOSKELETON OF HUMAN PLACENTAL MICROVILLI. John M. Bissonnette, Rolf L. Ingermann, Kent L. Thornburg, Patricia A. Koch and Karen M. Acott, Oregon Health Sciences University, Portland, Oregon 97201.

When viewed by thin section electronmicroscopy the microvilli of human placental synctiotrophoblast contain a core of filamentous structures consistent with actin microfilaments. Incubation of isolated microvilli with 0.7% Triton X-100, (8 mg detergent; mg protein) removed 80-85% of the membrane protein. The Triton insoluble material was recovered as a discrete fraction on density gradient centrifugation and when examined microscopically showed dimensions and forms similar to the intact microvilli without plasma membranes. Four major protein bands were resolved when the Triton insoluble fraction or cytoskeleton was examined by urea-sodium dodecyl sulfate polyacrylamide gel electrophroesis, one of which co-migrated with rabbit muscle actin. In addition, 6-8 minor bands were present. The Triton insoluble fraction was reconstituted with exogenous phospholipids and uptake of $|^{14}C|$-D-glucose and $|^{3}H|$-L-glucose measured by millipore filtration. D-glucose uptake exceeded that of L-glucose by 3-4 fold. Uptake by the Triton insoluble fraction without added phospholipids showed little distinction between D and L-glucose indicating that the observed transport by reconstituted cytoskeletons was not a function of intact membrane vesicles which had resisted the Triton extraction. These observations suggest that some of the D-glucose transporter is associated with the cytoskeleton of the placental microvillus. Supported by HD 12033 and HD 07084 from USPHS and MRF of Oregon.

0414 TEMPORAL AND SPATIAL MICROTUBULE REARRANGEMENTS MAY BE REQUIRED TO INITIATE CELL DIVISION, K.L.Crossin and D.H.Carney, Univ. of Texas Medical Branch, Galveston, TX 77550

We have recently shown that initiation of DNA synthesis by thrombin and epidermal growth factor in serum-free cultures of embryonic fibroblasts is inhibited by a one hour pretreatment of cultures with taxol, a drug which stabilizes microtubules (MTs) and prevents their disruption by cold or colchicine (Cell 27, 341-350, 1981). Addition of taxol at any time within 6 hours of growth factor addition inhibits as well as taxol added one hour prior to the growth factor; after 10 hours taxol no longer has an inhibitory effect on growth factor initiated DNA synthesis. To examine the nature of this temporal effect, we have studied the effects of reversible MT depolymerization by colcemid. A two hour exposure to colcemid transiently depolymerizes MTs and results in a two fold increase in DNA synthesis by 26 hours and a 1.5 fold increase in cell number by 48 hours. Four hr after colcemid removal MTs appear normal, thus an early transient depolymerization is sufficient to initiate DNA synthesis and cell division. This early signal can be blocked by MT stabilization at any time up to 8 hours after colcemid treatment suggesting that a second MT rearrangement at about 8 hours is critical for generation of a proliferative signal. To visualize the spatial arrangement of MTs in drug treated cells, we looked at antitubulin immunofluorescence following treatment with various concentrations of colchicine and taxol. The opposing effects of these drugs show a dose dependent correlation between MT depolymerization and initiation. In addition, concentrations of taxol which caused maximal inhibition of DNA synthesis stabilized MTs near the cell periphery suggesting that MT disruption near the cell surface may be sufficient to initiate DNA synthesis. (Supported by NIH Grant AM-25807).

0415 SERUM FACTORS AND CHLORPROMAZINE ALTER ACTIN CYTOSKELETAL ORGANISATION OF CULTURED MOUSE CELLS, David Alcorta, Scott Powers and Robert Pollack, Columbia University New York, New York 10027

Several mouse cell lines were examined for the responsiveness of their actin cable organization to a 48 hour exposure to the phenathiazine, chlorpromazine (CPZ), a calmodulin inactivator, and to the depletion of serum and hormone factors from the culture media. Cells were scored on an individual basis for their actin cable organization by the four class method of Verderame et al,PNAS 77:5624 ('80) using fluorescently labeled phalloidin .Fifty percent of early passage mouse embryo fibroblasts(MEF) cells had large cable classes I and II when grown in 10% Fetal Calf Serum (FCS).Addition of chlorpromazine to this medium exerted a time and concentration dependent effect upon the cell cable population coupled to cell rounding and detachment. At concentrations which did not effect adhesion no cable changes were seen.Stepwise serum concentration decreases down to 1.0 and 0.0% FCS increased the cable population to 65% and 85% respectively. Three to five micromolar chlorpromazine in 1% or no serum medium reversed these percentages to levels found in cells in 10% FCS with little cell detachment. The CPZ effect appears to specifically decrease the large cable as no effect is seen on cell lines such as 3T3-S1, a subclone of 3T3, and SV101, a SV40 transformant, which initially contain primarily fine class III cables. Treatment with chlorpromazine sulfoxide, an inactive analog showed no effects at similar concentrations. For 3T3, use of serum free media plus a hormone mixture revealed that the hormones were equivalent to high serum in keeping the cable percentages lower than cells incubated in low serum whereas deletion of insulin or epidermal growth factor were as effective as a total serum deletion in increasing the cable population. Additionally, 3T3 had similar concentration responsiveness to the cable disrupting effects of CPZ.

0416 SERUM-DEPENDENT LABILE PROTEIN IN NORMAL AND TRANSFORMED CELLS, Judith Campisi, Robert G. Croy and Arthur B. Pardee, Sidney Farber Cancer Institute, Boston, MA 02115.

When deprived of nutrients or serum factors normal cells arrest at a restriction point (R) in G_1, a few hours before S. Moderate inhibition of protein synthesis increases the time normal cells spend between mitosis and R (other parts of the cycle are unaffected); thus the hypothesis that serum-dependent accumulation of labile protein(s) is necessary for normal growth regulation. Partial inhibition of protein synthesis (50-80%) by cycloheximide (CHM) or histidinol temporarily prevented exponentially growing Balb/c 3T3 (A31) cells from entering S. Two tumorigenic derivatives, SV-40 transformed and benzo(a)pyrene transformed (BPA31), were much less affected. Complete inhibition of protein synthesis in A31 cells synchronized just prior to R delayed their entry into S for a period in excess of the the CHM pulse. This suggested a decay of labile protein, and we calculated a 2.5 hr half life. BPA31 cells showed no excess delay even with longer exposures (8 hrs) to CHM, suggesting that protein(s) required for progression are stable in these cells.

We have identified several polypeptides by 2D gel electrophoresis, using pulse-chase methods, which exhibit the temporal and kinetic patterns of the putative R protein(s). Three new proteins are synthesized shortly after serum addition to quiescent A31 cells. The major protein is labile with $t_{\frac{1}{2}}$<3 hrs; its rate of synthesis decreases before commencement of S. Five other new proteins are synthesized at various times after serum addition. The stability of these proteins in transformed cells is currently under investigation.

0417 CON A BINDING TO ERYTHROCYTES IS DEPENDENT ON CELL DENSITY. Julius A. Gordon, University of Colorado Health Sciences Center, Denver, CO 80262

Although the measurements on the binding of (labeled) native hormones, growth factors, and analogues to cell surface has increased our understanding of the ligand-receptor binding and its coupling with cellular event(s), the interpretation of the binding data may only be valid if some of the technical and theoretical problems are understood.

We have examined the role of cell density on ^{125}I-Con A binding to erythrocytes; a parameter generally avoided due to limitations imposed by methodology or materials. We found that increasing the erythrocyte concentration (1%-5%-10%) leads to a significant reduction in the Scatchard plot maximum (diminished positive cooperativity, decrease in the apparent Ka, and some apparent reduction in total number of acceptors). Care was taken to avoid the usual methodological problems by measuring free and bound ligand, varying the aliquot taken, and altering the amount and type of cushion in the Microfuge technique; labeled Con A was fully competitive with native Con A. Free Con A remaining after incubation with 10% native or fixed erythrocytes showed a marked reduction in binding ability with little or no loss of agglutinating ability, yet we found no obvious Con A degradation, loss of radiolabel, or selection when examined by gel electrophoresis; binding ability could not be restored following extensive dialyses.

The explanation for the binding dependence on cell concentration is not clear. It may involve an undetected processing of the ligand by the cell suspension, inhibition by shedding of cell acceptors or inhibitors, a cell density dependent cell to cell association, or any combination. (Supported by an NIH grant and a gift from the R.J. Reynolds Industries, Inc.).

0418 CHANGES IN MITOCHONDRIAL MEMBRANE POTENTIAL DURING CELL GROWTH STIMULATION BY GROWTH FACTORS AND DRUGS, Alan S. Waggoner, Visiting Prof. Pharmacology, Harvard Med. Sch. / Chemistry Dept.,Amherst College, Amherst, Mass. 01002.

Optical probes of membrane potential have proved useful for monitoring electrical activity in excitable tissues and for estimating membrane potentials of cells, organelles, and vesicles from numerous organisms. Recently, R. L. Cohen et.al., Nature,290, 593 (1981) and L. V. Johnson, J. Cell Biol. 88, 526 (1981) have reported that stimulation of growth-arrested cells in the presence of potential-sensitive fluorescent dyes produces a striking increase in the fluorescence of the mitochondria. These organelles accumulate the cationic, membrane permeant dye molecules probably because of an increase in the mitochondrial membrane potential after cell stimulation.

With Dr. J. Campisi and others in Dr. A. Pardee's laboratory and Dr. P. Horan at Rochester University Med Sch. I have been investigating the kinetics of mitochondrial energization of cultured cells following addition of growth factors and various drugs to quiescent cells. Using membrane potential probes we are also trying to quantify the magnitude of the mitochondrial and plasma membrane potentials of cells at different stages of the cell cycle.

The fluorescence measurements are being made on single cells by moving the cell culture dishes in a raster-scan pattern while using a helium-neon laser microspot as an illumination source for exciting potential-sensitive dyes.

0419 INDUCTION OF DIFFERENTIATION OF PROMYELOCYTIC LEUKEMIA CELLS BY AGENTS PRODUCING ALTERATIONS IN MEMBRANE PHOSPHOLIPIDS. Steven A. Fischkoff and Robert E. Gallagher, Baltimore Cancer Research Center, Baltimore, MD 21201

Alterations in the phospholipid composition of the cell membrane have been noted during the process of chemically induced differentiation in myeloid leukemia cell lines. Furthermore, inhibition of the incorporation of methyl groups into the polar head groups of cellular phospholipids appears to be an early biochemical event in the differentiative process. In order to better define the mechanistic significance of these observations, we incubated cultures of the human promyelocytic leukemia cell line, HL-60, with a variety of analogs of choline in serum-free medium consisting of RPMI 1640, insulin (5 µg/ml), transferrin (5 µg/ml), Hepes, penicillin and streptomycin. Dimethylsulfoxide and ethionine (both of which promote myeloid differentiation of HL-60 and the latter of which is an inhibitor of methylation) were also used as positive controls. We found that DMSO, N-methylethanolamine, N-ethylethanolamine, N-isopropylethanolamine, 2-aminopropanol, 2-methyl-2-aminopropanol and 2-aminobutanol induced myeloid differentiation. Ethionine and ethanolamine did so weakly, but choline, NN-dimethylethanolamine, and NN-diethylethanolamine possessed little, if any, inducing ability. Most of these agents produced changes (either positive or negative) in the incorporation of methyl groups, choline, ethanolamine, and glycerol into cellular phospholipids. Similarly, marked changes were seen in the distribution of arachidonic acid between various classes of phospholipids. However, none of these parameters correlated in a consistent way with a compounds' efficacy as an inducer. We conclude that compounds which produce alterations in cellular phospholipids may be effective at inducing myeloid differentiation, but the mechanism by which they do so remains unknown.

0420 RECEPTOR-MEDIATED MODULATION OF NORMAL AND LEUKEMIC HUMAN BLOOD CELL FUNCTION BY PHORBOL DIESTERS. J.B. Weinberg and B. J. Goodwin, VA Med. Ctr., Durham, NC 27705

Phorbol diester tumor promoters affect a variety of cells including normal and leukemic blood cells. The purpose of this study was to determine the binding characteristics of the phorbol diesters to blood cells using [20-3H]phorbol 12,13-dibutyrate (3HPDBu) and to correlate these findings with the different cellular responses. The dissociation constants (Kd) and the number of binding sites per cell were calculated using Scatchard analysis. All normal blood cells except rbc had specific, high affinity receptors for 3HPDBu. The binding was rapid, reversible, and specific. The dose effective in producing 50% of the maximal cellular response (ED50) (H202 generation by monos and PMN and TdR incorporation into lymphs) roughly correlated with the Kd for binding. The Kd for phorbol myristate acetate (PMA), determined by competition binding with 3HPDBu was 40nM in PMN, 18nM in monos, and 18nM in lymphs. This correlated with PMA's lower ED50 (16nM in PMN, 3nM in monos, and 18nM in lymphs).

	Lymphs	Monos	PMNs	Plts	ANLL	CLL	ALL
Kd:	50nM	37nM	24nM	31nM	76nM	112nM	96nM
#sites:	689K	663K	223K	17K	448K	320K	618K
ED50:	45nM	100nM	24nM	—	—	—	—

Cells freshly isolated from patients with acute nonlymphocytic leukemia (ANLL,n=13) differentiated into macrophage-like cells after culture with PDBu or PMA, while those from patients with chronic lymphocytic leukemia (CLL, n=4) and acute lymphocytic leukemia (ALL,n=5) did not. The differing responses were not caused by differences in the ligand binding, since their Kd's and numbers of receptors for 3HPDBu were comparable. Thus, normal and leukemic blood cell responses to the phorbol diesters, although qualitatively different, are mediated by comparable specific receptors.

0421 AZIDES OF PHLORIZIN AND PHLORETIN ARE POTENT INHIBITORS OF THE SUGAR TRANSPORTER IN THE HUMAN K-562 CML-DERIVED CELL. D.F. Diedrich, J.C. Dozier and S.J. Turco, University of Kentucky, Department of Pharmacology and Biochemistry, Lexington, KY 40536.

We have previously shown that human K-562 cells, known to possess erythropoietic characteristics, also possess a glucose transporter similar to the one present in adult human erythrocytes (J. Cell. Physiol. 108(1981)77); the zero-trans influx of 3-O-methylglucose (3-MG) was competitively blocked by phloretin and phlorizin (K_i = 4.1 and 225 μM, respectively). We now find that two derivatives of these inhibitors (phloretin-3'-benzylazide, PBAz, and phlorizin-5'-benzylazide, PhzBAz) are potent non-competitive inhibitors of [^{14}C]-3-MG zero-trans efflux from the K-562 cell (app. K_t = 7.8 mM; V_{max} = 23 nmoles/min/10^6 cells; Ki for PBAz and PhzBAz = 0.3 and 2.0 μM, respectively, not corrected for non-specific binding). Essentially the same inhibition constants were found for 3-MG equilibrium exchange in normal human erythrocytes, but under these transport conditions they act as strictly competitive inhibitors. Our interpretation of these findings is that both azide derivatives, particularly the glycoside, have high affinity for the transporter facing the outside of the cell. Our results suggest that the azides are potentially useful photolabeling agents for the sugar transporter which is present at a much greater density in the K-562 cell membrane than in the normal RBC.

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0422 EVOLUTION OF SPECIFIC HIGH AFFINITY RECEPTORS FOR BIOLOGICALLY ACTIVE PHORBOL AND INGENOL ESTERS, Mohammed Shoyab, Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, MD 21701

A variety of normal and transformed avian and mammalian cells and tissues contain high affinity specific receptors which interact with biologically active phorbol and ingenol esters in a reversible and saturable manner. The binding of labelled phorbol-12,13-dibutyrate (PDBu) to live or glutaraldehyde-fixed cells is dose-, time- and temperature-dependent. Those phorbol or ingenol esters which stimulate cell growth in culture and have tumour-promoting activity in vivo inhibit the binding of labelled PDBu, while the biologically inactive derivatives fail to do so. Other non-diterpene tumour-promoting agents, epidermal growth factor (EGF), retinoids and prostaglandins do not compete for the binding of labelled PDBu to its specific membrane receptors. PDBu binding sites are widely distributed in mouse tissues. Brain and spleen contain exceptionally high concentrations of these receptors. PDBu receptors are present in all regions of the brain. The Choroid plexus and pituitary bind markedly lower amounts of PDBu than other brain regions. Brains or spleens from different murine strains bind almost the same amounts of PDBu while those organs from embryonic mice bind very little PDBu. Binding increases with age and reaches a maximum at about 4 weeks of age. Young and mature brains from various species contain these specific binding sites. These data suggest a structural or enzymatic role for PDBu receptors.

0423 THE PHOSPHATIDYLINOSITOL CYCLE: EVIDENCE FOR A SEPARATE, HORMONE-SENSITIVE PHOSPHATIDYLINOSITOL POOL, Marie E. Monaco, New York University School of Medicine and the V.A. Hospital, New York, NY 10016

Phospholipid metabolism has been suggested as a possible candidate in mediation of the action of certain peptide hormones. In particular, phospholipid methylation and phosphatidylinositol (PI) turnover have been implicated. WRK-1 rat mammary tumor cells respond to vasopressin (VP) by a variety of biochemical and morphological alterations. Initially, there is a rapid increase in the incorporation of $^{32}P_i$ into PI, accompanied by a rapid loss of radioactivity from prelabeled PI. Observation of the effect of VP on loss of radioactivity (turnover of PI) is dependent upon the preincubation conditions. If cells are prelabeled acutely (30') with $^{32}P_i$ in the absence of hormone, then the resultant ^{32}P-PI is resistant to hormone-stimulated turnover. However, if VP is present during the lebeling period. then the $^{32}P_i$ which is incorporated into PI in response to VP (hormone-stimulated value minus control value) is fully sensitive to VP with respect to turnover. These data suggest the following: 1) the PI cycle in WRK-1 cells is closed, since the PI which is made during the synthetic phase in response to VP is subsequently turned over during the degradative phase; and 2) hormone-sensitive PI is somehow separate from that PI synthesized by the cell in the absence of hormone. In order to determine what percentage of the total cellular PI the hormone-sensitive moiety constitutes, cells were chronically prelabeled (5doublings) with $^{32}P_i$ and turnover subsequently monitored in the presence and absence of hormone. The results indicate the hormone-sensitive PI accounts for 17.1 $\pm$ 0.8% of the total cellular PI.

0424 STUDIES ON OPIATE RECEPTORS. Horace H. Loh, Depts. of Psychiatry and Pharmacology, University of California, San Francisco, CA. 94143.

Research is focussed primarily on membrane opiate and endorphin receptors. To characterize these receptors, we are studying them in the membrane as well as in the isolated form. Since it is known that opiate receptor is proteolipid in nature, we are trying to determine the exact role of membrane lipids on opiate receptor mechanism. (Our recent findings have been published in Ann. Rev. Pharmac. 20:201, 1980).

0425 EFFECTS OF FSH AND DIETARY VITAMIN E ON SERTOLI CELL PROSTAGLANDIN SYNTHESIS, D. R. Cooper & M. P. Carpenter, OK Med. Res. Fdn. & Dept. Bioc., OUHSC, OK City, OK 73104

Prostaglandins (PGs), products of arachidonic acid metabolism via the PG synthetase complex, are potent modulators of adenylate cyclase, cell differentiation, and proliferation. Both freshly isolated rat Sertoli cells (SC) and cells carried in short-term culture synthesize PGs. The age and dietary vitamin E-status of the rat from which these cells are isolated affect the types and levels of PGs produced. SC from 21 day old and 4 mo old rats were cultured for 3 days in modified Ham's F-12. Media was replaced and collected after 24 hr for PG analysis by RIA. Cells from 21 day old rats synthesize PGI_2. In the presence of FSH, PGE_2 is produced and PGI_2 production is stimulated 2-3 fold. Indomethacin blocks the FSH response. Using freshly isolated SC and tissue from SC-enriched (SCE) testes from fetally irradiated rats, this stimulation can be detected after 1 hr of incubation. $PGF_2\alpha$, a product of testis microsomes, is not detected in these preparations. SC cultures from adult rats synthesize PGI_2 and PGE_2. The total amount of PG (50 ng/mg/24 hr) is 3-5 fold higher than from 21 day old rats. SC from control diet rats synthesize 2-fold more PGE_2 than PGI_2. In cultures from vitamin E-deficient rats, less PGE_2 is synthesized ($PGE_2/PGI_2 \sim 0.7$). $PGF_2\alpha$ cross-reactive material is also present. Unlike PGE_2 and PGI_2, which are enhanced by FSH and inhibited by indomethacin, this material is not affected by either agent. Spermatogenesis is dependent upon dietary vitamin E. Vitamin E status and FSH affect SC PG synthesis in mature rats. FSH stimulates PG production in immature rats. Changes in PGI_2 and PGE_2 levels with FSH and dietary vitamin E have the potential to modulate SC functions and germ cell development as well as mediating effects of an adenylate cyclase activity. (Supported by NIH grant HD05641.)

0426 PLATELET MEMBRANE PROTEINS INVOLVED IN REGULATION OF FIBRINOGEN RECEPTORS, Elizabeth Kornecki and Stefan Niewiarowski, Temple Univ. Hlth. Sci. Ctr., Phila., Pa. 19140

Fibrinogen (fg) receptors remain latent on non-stimulated platelets. Treatment of platelets with ADP or chymotrypsin results in an exposure of two classes (low and high affinity) fg receptors and in platelet aggregation in the presence of fg. The rate of platelet aggregation depends on fg receptor occupancy. Experimental evidence suggests that three classes of platelet membrane components are involved in the exposure of fg receptors.(1) a 100,000 Mr component covalently labeled with 5'p fluorosulfonylbenzoyladenosine (FSBA), an ADP analogue; this component may protect fg receptor by a steric hindrance effect. Removal of the label from platelets by chymotrypsin restores their ability to aggregate. (2) Glycoprotein IIb (GPIIb) and glycoprotein IIIa (GPIIIa) are absent in Glanzmann's thrombasthenia, a condition in which ADP stimulated platelets do not bind fg and do not aggregate, however, anti-GPIIb and anti-GPIIIa antibodies do not block fg receptor mediated platelet aggregation. We postulate that GPIIb and GPIIIa are associated with low affinity fg receptor and are involved in the exposure of high affinity receptor. (3) a 70,000 Mr component is present in thrombasthenic platelets which also possess high affinity fg receptor exposed by chymotrypsin. This component is precipitated by polyclonal and monoclonal antibodies which block fg receptor mediated platelet aggregation. We hypothesize that high affinity fg receptors are associated with 70,000 Mr component of platelet membranes. (NIH HL-14217, 06356, 15226.)

0427 FEEDBACK INHIBITION OF ISLET CELL FUNCTION IN CHAMBERS. Francesco M. Marincola and Ronald C. Merrell, Stanford University School of Medicine, Stanford, CA. 94305

The role of physiologic cell relationships including cell-cell contacts and polarity with reference to a basal lamina has not been defined for islet cells. These relationships are drastically altered in culturing islet tissue for placement in chambers which sequester islet tissue from immune recognition. The basal lamina which separate individual islets from their mutual secretions is lost. The effect of reorganization on feedback inhibition was examined by studying the insulin output of equal aliquots of cultured islet tissue in a non-recirculating perifusion circuit where the endocrine products of one islet cell mass bathed the second. Pancreatic single cells were prepared from dogs by collagenase perfusion of the pancreatic duct. After three days in culture at 24°C. in a spinner flask the islet cells had formed neoislets which were distributed equally into two millipore chambers for circuited perifusion. Insulin secretion was measured at a port distal to each chamber at 37°C. with a flow rate of tissue culture medium at 0.7ml/min. containing either 5.5mM glucose (minimal stimulation) or 22 mM glucose (maximal stimulation). At minimal stimulation insulin output in both chambers was similar but when the insulin output of the first chamber exceeded about 30uU/ml during maximal stimulation the second chamber stopped secreting insulin in response to stimulation. These feedback effects must be considered in analyses of in vitro islet function and are important features in designing chambers for islet cell implantation.

0428 DIADENOSINE TETRAPHOSPHATE (Ap_4A) - A PUTATIVE SECOND MESSENGER OF PROLIFERATION CONTROL, Friedrich Grummt, Christa Weinmann, and Waltraud Albert, Institut für Biochemie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg, GFR.

Diadenosine tetraphosphate (Ap_4A) is ubiquitously distributed in eukaryotic cells. The intracellular concentration of Ap_4A fluctuates drastically in response to the proliferation rate, cell cycle phase and malignant transformation state. There is accumulating evidence that Ap_4A could play a role as a "second messenger" of cell cycle and proliferation control: 1) After mitogenic stimulation of G_1-arrested BHK and mouse 3T3 fibroblasts the Ap_4A pool gradually increased 1000 fold during progression through the G_1-phase reaching maximum Ap_4A concentrations (about 10 µM) in the S-phase. Cells lacking a defined G_1-phase (V79 lung fibroblasts, Physarum) possess a constitutively high basis level of Ap_4A. Ap_4A levels are high (1 µM) in mammalian embryos, in regenerating livers and developing sea urchin embryos. 2) The intracellular target of Ap_4A is DNA polymerase α. Ap_4A binds with high specificity and affinity ($K_D = 3 \times 10^{-7}$ M) to one of the subunits of the DNA polymerase holoenzyme. 3) Ap_4A triggers replicative DNA synthesis in vitro in G_1-arrested permeabilized BHK cells which is characterized by a high frequency of replication bubbles and synthesis of transient Okazaki fragment and their ligation.

0429 NORMAL MOUSE SERUM CONTAINS PEPTIDES WHICH INDUCE FIBROBLASTS TO GROWTH IN SOFT AGAR, Ulf R. Rapp and Mohammed Shoyab, National Cancer Institute, Frederick, MD 21701
The untransformed mouse fibroblast cells NIH/3T3, C3H/10T1/2 and the fibroblastic rat NRK cells do not grow in soft agar in medium supplemented with 10% fetal calf serum. When fetal calf serum in the growth medium was supplemented with sera from mice or other vertebrates, however, these cells were reversibly induced to form large colonies. Interestingly, the morphology of soft agar colonies was a function of the treated cell type, thus the fibroblastic NRK cells grew to smooth-surfaced spherical colonies, while C3H/10T1/2 cells grew as extended cells forming columns of end to end connected fibroblasts. We have purified to apparent homogeneity the growth promoting activity from mouse serum by gel filtration and high performance liquid chromatography (HPLC). The trypsin sensitive agar growth stimulating activity has an apparent molecular weight of 15-20 kilodaltons and elutes as a single peak from a C18-Bondapak/porasil column at 37% acetonitrile. Radioreceptor-competition experiments showed that the endogenous growth factor in the peak fraction did not compete for binding to PDBU receptors whereas it competed for EGF receptor binding. However, this peptide is different from EGF in its biological and biochemical properties. The novel serum growth factor retains biological activity in the nanogram range throughout the purification procedure. Thus, we have shown that normal sera from mice and also from other rodents and primates contain peptides with properties generally attributed to tumor promoting agents such as TPA. We conclude from this that such factors are necessary for normal tissue functions in the animal and may also play a role as cofactors in carcinogenesis.

Steroid Hormone Receptors: Genetic and Comparative Aspects

0430 THYROID HORMONE RECEPTORS IN A PRIMITIVE VERTEBRATE, Douglas S. Darling, and Aubrey Gorbman, University of Washington, Seattle, WA 98195

The diversity of intracellular thyroid hormone binding sites in mammals raises the question of which, if any, of these binding sites represent the general vertebrate pattern. A modified triiodothyronine (T3) binding assay was used with coho salmon (Oncorhynchus kisutch) liver nuclei. Purified nuclei were incubated with ^{125}I-T3 (or ^{125}I-thyroxine) with varied amounts of radio-inert thyroid hormone, or analogues. Bound hormone was seperated from free by use of polyethylene glycol. Salmon nuclei specifically bind T3 with a high affinity (K_a=1.031 $\pm$ 0.174 x 10^9 M^{-1}; $\bar{x} \pm$ S.E.; n=12) which is 50% of the affinity measured with rat liver nuclei with the same assay. Salmon nuclear T3-binding capacity is 0.127 $\pm$ 0.035 pmoles T3 per mg DNA (n=7). Rat liver nuclear capacity for T3 binding is 3.4 fold greater. Analysis of Scatchard plots indicates that the affinity of salmon hepatic nuclei for T3 is 10-fold greater than for thyroxine (T4). Based on plasma hormone concentrations, and nuclear binding affinities, T3 is calculated to be the major form of the hormone bound to salmon nuclei. The relative nuclear binding affinities for thyroid hormone analogues, as well as the affinity for T3, of salmon hepatic nuclei are very similar to human, rat, chick embryo, and larval amphibian hepatic nuclei. This suggests that the binding characteristics of the thyroid hormone receptor molecule have been highly conserved during vertebrate evolution.

0431 HORMONAL REGULATION OF HEPATIC ESTROGEN BINDING PROTEINS IN RATS, G.W. Lucier, T.C. Sloop, C.L. Thompson and R.C. Rumbaugh, NIEHS, Research Triangle, NC 27709
Recent studies have demonstrated that liver contains cytosolic estrogen receptor. Moreover, a second class of estrogen binding proteins have been identified which are termed higher-capacity lower-affinity (HCLA) binding sites. We have demonstrated that these binding proteins do not translocate estrogen to the nucleus but they do play a role in modulating nuclear translocation rates of cytosolic ligand receptor complex by altering availability of ligand for receptor. HCLA binding sites sediment in the 4S region of sucrose gradients, have a K_d of 10^{-8} M, bind both steroidal androgens and estrogens but not non-steroidal estrogens or other steroids. The levels of these sites undergo postpubertal sex differentiation such that adult male levels are 10-fold higher than observed in adult females or immature rats of either sex. Gonadectomy of adult rats has no effect on the levels of HCLA binding sites but neonatal castration of males prevents sex differentiation. Sex differentiation of HCLA binding sites can be imprinted by exposure to neonatal androgens during a critical period of postnatal development; administration of testosterone to castrate male rats between days 6 and 16 of life imprinted for male levels of HCLA sites. The imprinted effect is manifested postpubertally, is permanent and is pituitary dependent Male levels of HCLA sites can be imprinted in ovariectomized females by androgen treatment during the critical period. Estrogen treatment of male rats represses synthesis of HCLA sites. These studies demonstrate that the hypothalamic-pituitary-gonadal axis plays a key role in regulating synthesis of an important class of hepatic estrogen binding proteins.

0432 ECDYSTEROID RECEPTORS IN DROSOPHILA, Mary Alice Yund and David L. Osterbur, University of California, Berkeley, CA 94720

The insect steroid hormone, 20-hydroxyecdysone, is responsible for a complex series of morphological and biochemical changes throughout insect development. It is not known whether specificity of response at a particular developmental stage or in a particular tissue is due to differences in the hormone receptor or differences in other cell or tissue components. For several years we have studied ecdysteroid receptors in imaginal discs isolated from mid-third instar larvae of Drosophila melanogaster. Imaginal discs receptors are associated with nuclei and appear to reside on the chromatin before the increase in hormone titer in the late third instar that induces imaginal disc morphogenesis. We are examining the properties of imaginal disc receptors preliminary to the development of a scheme for purifying these receptors. The tissue specificity of ecdysteroid receptors is being investigated by a comparison of molecular and biochemical characteristics of receptors in third instar imaginal discs and in other tissues and developmental stages.

0433 JUVENILE HORMONE BINDING PROTEINS IN LEULOPHAEA MADERAE, John K. Koeppe and Gae E. Kovalick, University of North Carolina, Chapel Hill, NC 27514

In the ovoviviparous cockroach, Leucophaea maderae, juvenile hormone (JH) regulates specific processes and events in at least two different tissues during the reproductive cycle. In the fat body, JH appears to act like a steroid hormone to stimulate the synthesis and secretion of the major yolk protein, vitellogenin. In the follicle cells which surround the developing terminal oocyte, rates of DNA synthesis and the apparent synthesis of the enzyme thymidine kinase (TK) are both stimulated by JH.

Recent data from our laboratory demonstrates that JH-specific bonding proteins occur in both of these tissues as well as in the hemolymph. These binding macromolecules are prenase and heat sensitive and have a finite binding capacity for JH III. In the fatbody, the relative binding affinity (K_D) of this macromolecule for JH III is approximately [9 X 10^{-9}M], whereas the ovarian and hemolymph binding proteins have a K_D of around [2 X 10^{-8}M]. Competition studies, photoaffinity labeling of the macromolecules, and the properties of the de-lipidated macromolecules suggest these macromolecules are tissue specific.

0434 GENETIC AND EPIGENETIC BASES FOR GLUCOCORTICOID-RESISTANCE, Judith C. Gasson and Suzanne Bourgeois, The Salk Institute for Biological Studies, Regulatory Biology Laboratory, San Diego, California 92138.

A number of laboratories have shown that glucocorticoid receptor defects are the basis for glucocorticoid resistance induced by mutagenic treatments of glucocorticoid-sensitive lymphoid cell lines. We have used somatic cell genetics to analyze the basis for the glucocorticoid resistance of a cell line, SAK8, established in culture from a spontaneous AKR thymic lymphoma which exhibits resistance without mutagenesis.

Binding assays show that SAK8 cells contain approximately 30,000 glucocorticoid receptors per cell. These receptors appear functional since they complement receptor defects in hybrids and mediate a glucocorticoid-induced increase in the production of endogenous leukemia virus protein. Therefore, the SAK8 line is resistant to glucocorticoid-induced lysis because of a defect in one or several functions other than the receptor, designated "1" for lysis. Further genetic evidence for the "1" function(s) was obtained by chromosome segregation analysis in cell hybrids.

We have generated glucocorticoid sensitive clones of SAK8 by treatment with 5-azacytidine, a drug known to inhibit DNA methylation. This preliminary result suggests that it is possible to activate the "1" alleles which are not expressed in the SAK8 cells, and is consistent with the idea that resistance in SAK8 has an epigenetic origin, i.e., results from differentiation. (Supported by NIH fellowship #AM06179 and grant #GM20868 and by a grant from the Whitehall Foundation.)

0435 A CORTICOSTEROID BINDING PROTEIN AND LIGAND IN *C. ALBICANS*: A POSSIBLE STEROID-RECEPTOR SYSTEM, David Feldman and David Loose, Stanford University, Stanford, CA 94305

The evolution of hormone-receptor systems is presently undergoing intense investigation. It is not yet clear how early in evolution recognizable hormone-receptor systems developed. We have recently reported on the existence of a receptor-like binder in *Candida albicans* a unicellular, eukayotic fungus. Further characterization of this macromolecule now indicates it to be a corticosteroid cytoplasmic binder with high affinity, steroid selectivity and stereospecificity. Using [^{3}H]corticosterone as the radioprobe, competitive binding assays reveal the following binding sequence: corticosterone ≃ progesterone > cortisol > prednisolone >> dexamethasone; minimal affinity for estradiol, testosterone, aldosterone, or 1,25(OH)$_2$D$_3$ is exhibited. The apparent equilibrium dissociation constant (K_d) for corticosterone is 6 nM and the binding capacity is ∿ 700 fmol/mg cytosol protein. The binding is reversible and the [^{3}H]corticosterone appears unmetabolized. The binder sediments at ∿ 4S and has a molecular weight of ∿ 43,000. Binding is destroyed by heating to 56°C for 30 min or exposure to trypsin or N-ethylmaleimide. In addition, a lipid extractable material present in the fungal cells and released into the growth medium, reversibly competes for [^{3}H]corticosterone binding sites in fungal cytosol and appears to be the endogenous ligand for this binding protein. Of interest is the finding that the fungal extract also competes for mammalian glucocorticoid receptors. We hypothesize that the fungal binder and ligand may represent a primitive hormonal system. Furthermore, the demonstration of interactions between mammalian steroids and the fungal binder and between the fungal ligand and mammalian receptors suggests the possibility of important clinical consequences between host and potential pathogen.

0436 CELLULAR RECEPTORS FOR 20-HYDROXYECDYSONE AND JUVENILE HORMONE IN INSECT EPIDERMIS, Lynn M. Riddiford and Karen A. Dyer, University of Washington, Seattle, WA 98195

Lepidopteran epidermis produces first a larval, then a pupal, then an adult cuticle. The larval cuticle is made in response to 20-hydroxyecdysone (20HE) in the presence of juvenile hormone (JH); the switch to pupal cuticle production occurs when 20HE acts in the absence of JH. We have been studying the biochemical properties of the cellular receptors for both hormones in larval epidermis. Both are rapidly taken up by the cells ($t_{1/2}$ ≃ 5 min) into the cytoplasm, and small amounts are translocated to the nucleus. Nuclear receptor-assays revealed $k_d = 1.8 \times 10^{-8}$M for JHI in larval epidermis and the absence of specific binding of JHI in pupally-committed epidermis. Similar studies are underway for 20HE. EM-autoradiography of lysed nuclei shows 20HE is bound to multiple sites on the matrix as well as to individual sites on chromatin fibers. Radioactive hormone is found only over nuclear material and remains localized whether the chromatin is condensed, moderately dispersed, or dispersed completely into 10 nm strands. Not all nuclei in this target tissue appear to bind the hormone equally. Supported by grants from NIH and the Whitehall Foundation.

Hormone Receptor: Gene Interactions

0437 ESTROGEN INDUCTION OF AN EARLY mRNA SPECIES IN ESTROGEN RESPONSIVE HUMAN BREAST CANCER CELLS IN VITRO. Lee F. Allen, Robert G. Wilson, and Jolanta J. Cholon,Departments of Pathology and Biochemistry,CMDNJ,New Jersey Medical School, Newark, N.J. 07103.

It is well established that hormones modulate the growth of some human breast cancers and hormone responsive breast cancer cells in vitro (i.e. MCF 7). An attempt was undertaken in this laboratory to identify, through cell cycle manipulation, the induction of an estrogen specific mRNA species in estrogen responsive MCF 7 cells; a mRNA species which may modulate hormone action. For experiments, cells were grown in medium supplemented with hormone stripped serum supplemented with physiological concentrations of hormone. One day prior to experiments, the hormones were removed in order to free receptor proteins and allow for maximal stimulation. One half the cultures were stimulated by the addition of estradiol and both groups were simulateneously labeled with (^{3}H) adenosine for one hour. Cytoplasmic RNA was extracted from magnesium precipitated polysomes by SDS/ phenol/ chloroform extraction procedures, and poly(A) containing species were separated by poly(U) sepharose column chromatography, eluting with increasing concentrations of formamide. The mRNA species were fractionated by agarose gel electrophoresis and assayed for radioactivity through liquid scintillation counting. Our results show that if proper conditions are provided, estrogen indeed is able to stimulate the induction of a specific mRNA which predominates in the first hour following hormone addition. This points strongly to the regulatory function of this specific mRNA in the hormonal regulation of the growth of hormone dependent human breast cancer cells. Supported by a grant from the Foundation of CMDNJ.

0438 PARAMETERS AFFECTING THE SPECIFIC BINDING OF 3-METHYLCHOLANTHRENE TO HEPATIC CYTOSOLIC PROTEINS AND THEIR SUBSEQUENT INTERACTION WITH DNA. Brian Tierney, Glenn Bailey and Edward Bresnick, University of Vermont College of Medicine, Burlington, Vermont 05405

Recent studies have indicated that the cytochrome P-450c enzyme detoxification system may be mediated via a cytosolic receptor protein. We have found specific binding to rat liver cytosolic proteins when the cytochrome P-450c inducer, 3-methylcholanthrene (3-MC) was employed as ligand. Such binding was of high affinity (K_D 2.54 nM) and saturable. The specific binding is pH and temperature dependent. The 3-MC binding proteins were examined on DNA-cellulose and only liver extracts from cytochrome P-450c-inducible animals contained the binding proteins. Apparent temperature-dependant activation of the cytosolic protein/3-MC complex on binding to DNA-cellulose was observed. This binding was also enhanced by the addition of ATP and magnesium. (Supported by CA-20711).

0439 Oligodeoxynucleotide Base Recognition by Steroid Hormone Receptors, Herbert W. Dickerman and S. Anand Kumar, New York State Dept. of Health, Albany, N.Y. 12201

Oligodeoxynucleotides covalently linked to cellulose were used as probes of the DNA binding domains of mouse steroid holoreceptors: uterine and kidney estradiol (E_2R), liver dexamethasone (dexR) and kidney testosterone receptors. With uterine E_2R, the relative binding order was oligo(dG) > oligo(dT) $\geq$ oligo(dC) >> oligo(dA) > oligo(dI). The binding reactions were salt-sensitive with the optimal KCl concentration of 0.1 - 0.2M. There was no enhancement of binding by activation, either temperature or salt induced. Quantitative differences in oligonucleotide binding were elicited with pyridoxal 5-phosphate, Cibacron blue F3GA and diethylpropocarbonate. These, and qualitative differences following heat and purification, led to a designation of 2 types of subsites within the DNA binding domain of uterine E_2R. These are stable G sites which interact with oligo(dG) and labile N sites which bind to oligo(dT), oligo(dC) and oligo(dA). Stimulation of E_2R binding to N sites and stabilization of the holoreceptor was effected by histones H2A and H2B as well as the N terminal half molecule of H2B. The liver dexR required activation for optimal binding to the oligonucleotides, showed a marked responsiveness to salt concentration in the binding reaction and clearly had better binding to oligo(dT) > oligo(dC). The kidney testosterone receptor binding did not require salt activation, was insensitive to salt stimulation, and while the poorest binding receptor, showed a relatively higher binding to oligo(dA). The above data indicates that steroid holoreceptors can discriminate structural features of deoxynucleotide bases and this recognition process can be modulated by accessory proteins. (Supported by HHS/PHS grant AM23075)

0440 CHICKEN OVIDUCT PROGESTERONE RECEPTOR : LOCATION OF SPECIFIC REGIONS OF HIGH AFFINITY BINDING IN CLONED DNA FRAGMENTS OF HORMONE RESPONSIVE CHICKEN GENES, Eileen R. Mulvihill, Jean-Paul LePennec and Pierre Chambon, Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Faculté de Médecine, 11 Rue Humann 67085 STRASBOURG Cédex FRANCE.

We have used a DNA-cellulose competitive binding assay similar to that described by Kallos and Hollander (1978) to measure the extent of displacement of chick oviduct progesterone receptor complex from calf thymus DNA-cellulose by purified, cloned fragments of genomic DNA. Several DNA fragments from hormonally responsive genes coding for egg-white proteins, were found to be efficient competitors for either crude or partially purified receptor complexes when compared with calf thymus DNA. Data obtained using deletion mutations constructed in vitro allowed deliniation of a specific region necessary for strong competition, located 250-300 bp upstream from the mRNA startsite of the ovalbumin gene. Sequence homologies with this 5' upstream region were observed in other fragments of the ovalbumin, conalbumin, ovomucoid, X and Y genes which were also efficient competitors. Based on a comparison of such sequences of homology a consensus sequence which may constitute a progesterone receptor complex binding region has been constructed :
A T C C/T C/T A T T A/T T C T G G/T T T G T A. The results suggest that specific double-stranded DNA sequences are recognized by the oviduct progesterone receptor complex in vitro and are relevant to the question of whether or not specific DNA sequences are directly involved as genomic binding sites for steroid receptors.

0441 HMG AND H1 BINDING SITES ON NUCLEOSOMES, Ronald L. Seale, Department of Cellular Biology, Scripps Clinic and Research Foundation, La Jolla, California 92037

Monomeric nucleosomes have independent binding sites for H1 histones and for HMG proteins. The two types of monomers have been selectively fractionated from nuclease-digested chromatin, and show distinct electrophoretic mobilities. When H1 or HMG proteins are removed by dissociation in NaCl, they can be faithfully reconstituted onto the stripped nucleosomes. The possibility that H1-nucleosomes preferentially reassociate with H1, and that HMG-nucleosomes preferentially reassociate with HMG proteins was tested by reconstitution of each type of stripped particle with varying mixtures of H1 and HMG proteins. The individual stripped particles, although possessing both binding sites, preferentially reassociated with the type protein which was originally bound.

0442 ESTROGEN INDUCTION OF AN EARLY mRNA SPECIES IN ESTROGEN RESPONSIVE HUMAN BREAST CANCER CELLS IN VITRO. Lee F. Allen, Robert G. Wilson, and Jolanta J. Cholon, Departments of Pathology and Biochemistry, CMDNJ, New Jersey Medical School, Newark, N.J. 07103.

It is well established that hormones modulate the growth of some human breast cancers and hormone responsive breast cancer cells in vitro (i.e. MCF 7). An attempt was undertaken in this laboratory to identify, through cell cycle manipulation, the induction of an estrogen specific mRNA species in estrogen responsive MCF 7 cells; a mRNA species which may modulate hormone action. For experiments, cells were grown in medium supplemented with hormone stripped serum supplemented with physiological concentrations of hormone. One day prior to experiments, the hormones were removed in order to free receptor proteins and allow for maximal stimulation. One half the cultures were stimulated by the addition of estradiol and both groups were simultaneously labeled with (^{3}H) adenosine for one hour. Cytoplasmic RNA was extracted from magnesium precipitated polysomes by SDS/ phenol/ chloroform extraction procedures, and poly(A) containing species were separated by poly(U) sepharose column chromatography, eluting with increasing concentrations of formamide. The mRNA species were fractionated by agarose gel electrophoresis and assayed for radioactivity through liquid scintillation counting. Our results show that if proper conditions are provided, estrogen indeed is able to stimulate the induction of a specfic mRNA which predominates in the first hour following hormone addition. This points strongly to the regulatory function of this specific mRNA in the hormonal regulation of the growth of hormone dependent human breast cancer cells. Supported by a grant from the Foundation of CMDNJ.

0443 CHROMOSOME MAP ASSIGNMENT OF HUMAN SURFACE RECEPTORS: THE 4F2 DETERMINANT

M.E. Kamarck, P. Messer-Peters and F.H. Ruddle, Yale University, New Haven, Conn. 06511

The monoclonal antibody 4F2 identifies a human cell surface antigen expressed on activated B and T cells (Haynes et al., 1981) as well as on log phase fibroblasts. As a blast specific marker, the 4F2 determinant is the first to appear in mixed-lymphocyte culture and has the longest temporal expression. It thus seems likely that the 4F2 antigen defines a hormone receptor.

We have mapped the locus which specifies this determinant using a novel combination of somatic cell genetics and fluorescence-activated cell sorting (FACS II). Two human x mouse hybrid lines which accounted for all human chromosomes were used in this study. These hybrids, heterogeneous for 4F2 antigen expression, were sorted to yield populations homogeneous with respect to the presence or absence of this determinant. Isozyme analysis of these lines, intended to identify the subset of human chromosomes remaining in each population, indicated that only chromosome 11 segregated concordantly with 4F2 antigen expression. This map assignment was confirmed using a hybrid line which contained only human chromosome 11 on a mouse genetic background. This methodology will allow the rapid and efficient chromosome assignment of loci coding for any human surface receptor for which a specific antibody is available.

0444 INTERACTION OF THYROID HORMONE RECEPTOR WITH CHROMATIN COMPONENTS. Andrew J. Perlman*, Frederick Stanley, and Herbert H. Samuels. NYU Med. Center, N.Y., N.Y. 10016.

In previous studies we have shown that digestion of GH_1 cell nuclei with micrococcal nuclease releases the receptor as a major 6.5 S form which likely reflects an interaction of receptor with linker DNA. DNase I converts the 6.5 S form to slower sedimenting forms which attain a limit size of 3.8 S identical to the 3.8 S receptor extracted from nuclei by 0.4 M KCl. We have further characterized the structure of the 6.5 S form. The 6.5 S form does not bind duplex DNA while the smaller forms are DNA binding species, indicating that DNA may be a component of the 6.5 S species. Using ultracentrifugation in D_2O containing gradients and Sepharose CL-6B column chromatography we have determined the density (ρ), Stokes radius (R_s), and frictional ratio due to shape $(f/f_o)_{shape}$ for the 6.5 S and 3.8 S forms. For the 3.8 S form: M_r = 54,000, R_s = 3.3 nm, ρ = 1.36 g/cm^3, $(f/f_o)_{shape}$ = 1.212 indicating a relatively globular structure. For the 6.5 S form: M_r = 149,000, R_s = 6.0 nm, ρ = 1.42 g/cm^3, $(f/f_o)_{shape}$ = 1.36, indicating a more asymmetric form. The density of the 3.8 S form is characteristic of protein, and assuming that the density increment of the 6.5 S form reflects an associated DNA fragment the 6.5 S species was calculated to be composed of 85% protein and 15% DNA, equivalent to 36 base pairs. This was further verified by specific regeneration of the 6.5 S form from a salt extract of nuclei and specific size (40 ± 10 BP) duplex DNA fragments produced by micrococcal nuclease digestion. The results suggest that the receptor may exist as an oligomer in chromatin or in association with other discrete proteins complexed with DNA.

0445 HORMONAL REGULATION OF HUMAN TESTOSTERONE-ESTRADIOL-BINDING GLOBULIN SYNTHESIS, David P. Aden, Barbara B. Knowles and William Rosner*, The Wistar Institute, Philadelphia, PA 19104 and *Columbia University, New York, NY 10019

The human liver-derived cell lines Hep 3B and Hep G2 synthesize and secrete numerous plasma proteins and exhibit many other normal liver parenchymal cell functions. The synthesis of certain plasma proteins by these cell lines has been demonstrated to be controlled. For example, alpha-fetoprotein is under growth cycle control (D.P. Aden, et al., Nature 282:615) and the synthesis of apolipoprotein B can be inhibited by the addition of albumin to the culture medium and then restored to normal with the addition of oleic acid (J.M. Rash et al., Biochem. Biophys. Acta., in press). We have recently established that the Hep G2 cell line additionally secretes testosterone-estradiol-binding globulin (TeBG). TeBG exists as a trace protein in human plasma where it binds certain estrogens and androgens with high affinity and thus regulates the free concentration of these hormones. We have now demonstrated that the synthesis of TeBG by these cells can be regulated by the addition of estradiol, thyroxine or tamoxifen citrate. The regulation of TeBG synthesis also appears to be specific, for the same cultures do not produce increased amounts of other plasma proteins.

0446 METHYLATION AND DNASE 1 SENSITIVITY OF SPECIFIC GENES, Don J. Diamond and Howard M. Goodman, Massachusetts General Hospital, Boston, Mass 02114

We have examined the state of methylation of both the rat growth hormone(gh) and insulin genes in a variety of tissue culture cells and organs. Together these results suggest that in some cases methylation or DNASE 1 sensitivity is not rigidly associated with the "ON" state of a gene. The methylation pattern of the gh gene shows little correlation with its state of expression. However, in GH-3 cells the protein secretion into the medium and the level of gh-specific RNA can be induced approximately 50x from the basal level(serum substitute) with the addition of dexamethasone(1 uM) and thyroxine(10 nm). DNASE 1 studies of nuclear preparations from two rat cell lines (GH-3 and HTC) show small differences in the sensitivity of the gh gene in an expressed or non-expressed state. We have also examined the state of methylation of the rat 1 and 2 insulin genes in a B-cell Insulinoma. Both Insulin genes are almost completely hypomethylated within the tumor DNA, although the gh gene is fully methylated as in other tissues. Recently we have examined purified Islet DNA (B-cell enriched) and we have found only limited differences in the methylation pattern from non-Islet pancreatic tissue.

0447 VARIANT HUMAN BREAST CANCER CELLS THAT SYNTHESIZE LARGE AMOUNTS OF PROGESTERONE RECEPTORS DESPITE ESTROGEN AND ANTIESTROGEN RESISTANCE. K.B. Horwitz, University of Colorado Health Sciences Center, Denver, Colorado 80262.

In all estrogen targets studied to date, progesterone receptors (PgR) are synthesized under control of estradiol, acting through estrogen receptors (ER). We have developed and studied a variant of T47D human breast cancer cells which synthesize extraordinary amounts (300,000 sites per cell) of PgR in the absence of estradiol, and in the presence of high concentrations (1 μM) of the antiestrogen nafoxidine. The cells are estrogen and antiestrogen resistant as shown by growth studies and PgR synthesis. The ER are anomalous: there are no unfilled ER in cytoplasm or nuclei; only filled nuclear receptors are present which appear to be in a persistently activated, or "processed" state. We have studied the binding, translocation, nuclear turnover and replenishment of PgR in detail following progesterone treatment: cytoplasmic depletion is progestin specific; nuclear translocation is rapid (1-2 min) and stoichiometric; nuclear turnover is extensive (∿ 80% total cell PgR are lost in 30-60 min) but chloroquine (100 mM) inhibitable; replenishment is protein synthesis dependent but estrogen independent. We have purified and characterized the human breast tumor PgR by standard techniques and by photoaffinity labeling: receptors consist of two dissimilar subunits whose MW is 108K and 79K; only one subunit binds DNA-cellulose. Despite their resistance to estradiol and antiestrogen, PgR are not constitutively synthesized; BudR (20 μg/ml) and butyrate (10 mM) can selectively inhibit PgR production. In sum, these variant cells have anomalous ER, their PgR are estrogen independent yet they retain some characteristics of inducible proteins.

0448 DISSOCIATION OF STEROID BINDING TO RECEPTORS AND STEROID INDUCTION OF BIOLOGICAL ACTIVITY IN A GLUCOCORTICOID RESPONSIVE CELL, S. Stoney Simons, Jr. (NIADDK), Louis Mercier (NIADDK), and E. Brad Thompson (NCI), National Institutes of Health, Bethesda, MD 20205

Glucocorticoid responses in two independently derived lines of rat hepatoma tissue culture cells (HTC and FU5-5) were examined. FU5-5 cells exhibited induction of the enzyme tyrosine aminotransferase (TAT) at concentrations of dexamethasone that were ~ 7-fold lower than that required for HTC cells. This increased sensitivity of FU5-5 cells was not, however, due to an increased affinity of FU5-5 cell receptors for dexamethasone, as determined from cell-free and whole cell binding experiments. The inter-cell line difference in steroid sensitivity for TAT induction was observed with three other structurally different glucocorticoids, thus apparently ruling out steroid metabolism in one of the cell lines as a cause. Induction of TAT in FU5-5 cells was also observed to occur at ~ 8-fold lower steroid concentrations than was required for the expression of another glucocorticoid-inducible function (i.e., glutamine synthetase [GS]) in the same cells. Thus the dose-response curves for TAT induction in HTC cells and for GS induction in FU5-5 cells are closely correlated with whole cell steroid binding while the dose-response curve for TAT induction in FU5-5 cells is shifted to lower steroid concentrations. This represents the first report of dissociation of two supposedly primary, glucocorticoid-induced functions and indicates that identical receptor-mediated mechanistic processes cannot be utilized by FU5-5 cells for the induction of TAT and GS. The involvement of second messengers or different nuclear processes are possible explanations for the unusual behavior of FU5-5 cells during glucocorticoid induction of TAT.

Steroid Hormone Receptor Activation and Alteration

0449 NUCLEAR "PROCESSING" OF ESTROGEN RECEPTOR REPRESENTS CONVERSION TO A NEW RECEPTOR FORM, J. Strobl*, A. Kassid†, and M. Lippman†, *Laboratory of Biochemistry, †Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205

The mechanism of the apparent disappearance of nuclear estrogen receptors ("receptor processing") in MCF-7 human breast cancer cells which is correlated with estrogen induction of progesterone receptors and cell growth is unknown. To address this issue, we have performed kinetic and equilibrium binding studies of competible [^{3}H]estradiol binding in MCF-7 cells after 1 and 6 h of exposure to [^{3}H]estradiol. We find, in contrast to previous investigations, that high affinity, slowly dissociating nuclear estradiol receptors do not disappear following exposure to estradiol. Instead, these receptors are converted within the nucleus to a modified form which less readily exchanges bound estradiol and are KCl-extractable only in combination with sonication. Thus, some estrogenic effects in MCF-7 cells are dependent upon the further intranuclear conversion of the activated, translocated nuclear receptor to the "processed" receptor form. We also find that 50% of a lower affinity, more rapidly dissociating class of estradiol binding sites do disappear following estradiol exposure. Loss of these binding sites and processing of estrogen receptors proceed with a similar time course and both are inhibited by actinomycin D but not ethidium bromide. We suggest that receptor processing and this additional post-nuclear translocation event are related and reflect nuclear events which are crucial for elicitation of estrogenic responses in MCF-7 cells.

0450 ACTIVATION OF GLUCOCORTICOID-RECEPTOR COMPLEX, G. Litwack, T.J. Schmidt and C.A. Barnett, Fels Research Institute, Temple University School of Medicine, Philadelphia,PA.

We refer to activation(act'n) as the process by which the glucocorticoid-receptor complex is converted from a form which cannot bind to nuclei or to DNA to a form which has these capabilities. Unactivated (GRu) and activated (GRa) complexes have been separated on DEAE resins and can be quantified by this procedure as well as by DNA-cellulose or nuclear binding. Following i.p. injection of [^{3}H]triamcinolone into adrenalectomized rats, a progression in the appearance of GRu and then GRa is observed suggesting a precursor-product relationship and that the act'n process has physiological significance. The properties of GRu and GRa are similar whether formed in vivo or in vitro. Some insights into the act'n step come from the abilities of certain substances to stimulate or inhibit the rate of act'n. Various phosphorylated compounds such as triphosphates, like ATP, glycer-P or the addition of bovine alkaline phosphatase (AP) or other substrates of AP action stimulate act'n.Molybdate, tungstate and vanadate, known phosphatase inhibitors, inhibit act'n while o-phenanthroline, a chelator of heavy metal ions, blocks the binding of GRa to DNA-cellulose. Endogenous inhibitors of act'n and DNA-binding exist in liver cytosol. "Modulator" refers to an apparent small molecule which inhibits the actn step. Another small molecule inhibits DNA-binding and this appears to be pyridoxal-P which forms a Schiff base with a lys residue exposed on the GRa. Other chemical probes indicate that GRa but not GRu contains a group of positive charges, consisting minimally of arg, lys and his residues required for binding to DNA or nuclei. Protease inhibitors do not influence the act'n step. Collectively, the act'n step may involve dephosphorylation, dissociation of modulator and appearance of a group of positive charges essential for activity of GRa.

0451 **INDUCTION OF ESTROGEN RECEPTOR IN THE HAMSTER UTERUS BY CHOLERA TOXIN**
James G. Kenimer, John J. Alleva, and Alexander W. Jordan, Division of Drug Biology, Food and Drug Administration, Washington, D.C. 20204

Cholera toxin (CT) induces stromal edema and mucosal mitosis in the uterus of long-term ovariectomized hamsters. These are established effects of estrogen in rodents. Thus, CT has estrogen-like effects on the hamster uterus (Alleva and Lamanna, Annual Meeting of the Endocrine Society, 1979, Abstract 544). Since estrogen exerts its effects via interaction with its cytoplasmic receptor followed by translocation of the estrogen-receptor complex into the nucleus, we have examined the effect of CT on hamster uterine receptor for estrogen in cytoplasm (cRe) and nucleus (nRe). CT was injected into the right uterine horn and its solvent into the left horn on Day 1 (day of ovulation) of the 4-day estrous cycle and the hamsters were killed 2, 24, and 48 h later. Levels of cRe (fmol/mg DNA) in the right horn at these times were increased 0, 47, and 150 % above left horn levels; nRe levels were increased 0, 128, and 72 %. After intraperitoneal injection of CT into long-term ovariectomized hamsters cRe levels at 1, 3, 5, 7, 18, 24, and 48 h after injection were 1, 5, -9, 32, 124, 152, and 478 % of control levels; values for nRe were -58, 10, -3, -18, -10, 35, and 606 %. Estrogen administered to ovariectomized hamsters pretreated with CT resulted in translocation of cRe to the nucleus. The increase in cRe may explain the estrogen effects of CT and suggests the involvement of cyclic AMP in the induction of estrogen receptor.

0452 AN EQUILIBRIUM MODEL FOR GLUCOCORTICOID RECEPTOR ACTIVATION AND TRANSLOCATION, Bruce M. Raaka and Herbert H. Samuels, New York Univ. Med. Ctr., New York, NY 10016

The glucocorticoid receptor in GH$_1$ cells cultured without glucocorticoids and harvested in hypotonic buffer is primarily a 10 S cytosolic protein. When cells are cultured for 1 h at 37° with varying concentrations of [^{3}H]triamcinolone acetonide (TA), a 4 S receptor form is also found in the cytosol. Total cytosolic receptor decreases with increasing concentrations of [^{3}H]TA and a corresponding increase in nuclear receptor occurs. The distribution of cytosolic receptor in 10 S and 4 S forms shifts toward the 4 S form with increasing hormone concentrations. The 10 S form can be converted to the 4 S form in cytosol in vitro by heating with hormone at 25° or by raising the ionic strength at 4°. Molybdate (25 mM) inhibits these in vitro conversions and also inhibits 10 S to 4 S conversion and nuclear translocation in whole cells incubated at 25° with 15 nM [^{3}H]TA. Dense amino acid labeling studies suggest that the 10 S receptor form is an oligomer which at 37° is in a rapidly-exchanging equilibrium with its 4 S subunit. When cells are cultured for 1 h at 37° with 15 nM [^{3}H]TA, about 70% of the total receptor is found in the nucleus. If the cells are then incubated without hormone for 2 h at 37°, almost all of the receptor reappears in the cytosol in the 10 S form. Density labeling indicates that this reappearance is not due to new receptor synthesis. Thus, both 10 S to 4 S conversion and nuclear translocation of receptor are reversible. A model for hormone-mediated receptor translocation is proposed in which binding of hormone shifts the equilibrium between the 10 S and 4 S forms toward the 4 S form. The 4 S cytosolic form is a DNA binding protein and is in equilibrium with 4 S receptor bound to chromatin.

0453 THE ROLE OF ESTROGEN RECEPTOR PROTEINS IN THE INHIBITION OF HORMONE RESPONSIVE BREAST CANCER CELLS BY AMINONUCLEOSIDE OF PUROMYCIN. Jolanta J. Cholon, Robert W. Lockwood and John Mallams, Departments of Pathology and Radiology, College of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, N.J. 07103.

Recent results in this laboratory indicate that aminonucleoside of puromycin differentially affects hormone responsive as opposed to hormone unresponsive human breast cancer cells. It selectively inhibits DNA synthesis and proliferation of hormone responsive but not hormone unresponsive cells. We, therefore, sought to establish whether this effect is exerted through the estrogen receptor mechanism present in hormone responsive cells. For experiments, cells were grown on medium containing hormone stripped serum supplemented with physiological concentrations of hormones with the exception of estrogen. The cells were then stimulated by the addition of estrogen and half the cultures were treated with aminonucleoside of puromycin. The cells were harvested at periodic intervals following aminonucleoside treatment, homogenized and assayed for estrogen receptor proteins by sucrose gradient centrifugation. The results show that aminonucleoside has no effect on estrogen receptor protein interaction indicating that the inhibition of hormone responsive cells by aminonucleoside is not mediated through estrogen receptor proteins. In conclusion, these results suggest that aminonucleoside of puromycin inhibits hormone responsive human breast cancer cells through a mechanism independent of estrogen receptor proteins, which is particular to these cells and which represents another characteristic distinguishing hormone responsive from hormone unresponsive breast cancer cells. Supported by a grant from the Foundation of CMDNJ.

0454 A HORMONE-RESPONSIVE COLLAGENASE OF HUMAN BREAST CARCINOMA CELLS, Richard Kao, Mitchell Wong, and Robert Stern, University of California, San Francisco, CA 94143

Collagenase is secreted into the media of the cultured human breast tumor cell line ZR 75-31A. This activity, assayed by the Johnson-Wint procedure (Anal. Biochem. 104:175, 1980) measures the hydrolysis of ^{3}H-labeled collagen gels in the wells of microtiter plates. Cells were grown in the absence of serum for 48 hours after the addition of hormone. A 100-fold increase in activity was observed following the addition of either androstanolone ($10^{-9}M$) or hydrocortisone ($10^{-6}M$) compared to cells grown without hormone. No increase in activity was found after the addition of estradiol, dexamethasone, progesterone, insulin, or testosterone. No activity was found in the media of human fibroblasts or Hela cells grown in either the absence or presence of hormones. The scirrhous reaction to human breast cancer is composed primarily of collagen. Much of the actual tumor mass is due to this fibrotic response. The present findings indicate that the breast tumor cells themselves may modulate collagen tunrover. This may in part be the mechanism for the observed reduction in tumor mass following endocrine therapy for breast cancer. (Supported by USPHS NIH Grant CA 25179)

0455 MONOCLONAL ANTIBODIES TO ESTROGEN RECEPTORS, Bruno Moncharmont and Indu Parikh, Wellcome Research Laboratories, Research Triangle Park, NC 27709

Monoclonal antibodies that react with estrogen receptor have been prepared by hybridization of mouse myeloma cell and spleen cells of a mouse immunized with native receptor purified from calf uterus. The receptor specific immunoglobulins secreted by the hybrids were of IgG class and were able to bind to staphylococcal protein A. The dissociation constant (Kd) of the receptor-antibody complex was determined either in solution (0.56 nm) or in solid phase (0.05 nm). The ability of the antibodies to interact with the receptor was also studied by sucrose density gradient analysis. Depending on the concentration of the antibody present, antibody-antigen complexes of different sizes are formed. At lower antibody concentration the receptor-antibody complex sediments as 12.S and as 7.5.S at higher antibody concentration. This indicates that depending on the antibody concentration one or two sites of the IgG molecules are occupied and that only one accessible antigenic determinant is present in each 4S receptor molecule.

0456 ANTI-ESTROGEN RESISTANT VARIANTS OF HORMONE RESPONSIVE MCF-7 HUMAN BREAST CANCER CELLS, M.E. Lippman, H. Nawata, D. Bronzert, F. Vignon and H. Rochefort, Medical Breast Cancer Sec, N.C.I, Bethesda, Md; Endocrinology Unit, INSERM U148, Montpellier, France

To further clarify our understanding of the mechanism of action of estrogens (E) and anti-estrogens (AE) we have developed and partially characterized hormone independent variants of MCF-7 human breast cancer cells derived from the hormone responsive parental line. Wild type MCF-7 cells were cloned in soft agar in the presence of 10^{-6}M Tamoxifen. Two AE resistant clones have been studied. Both are identical to MCF-7 by chromosomal analysis and allozyme phenotype. Both contain ER with normal affinity, specificity, subcellular localization and sucrose density gradient behavior. One clone, R3 shows minimal residual inhibition of growth and macromolecular synthesis by AE. R3 has no progesterone receptor (PgR) which normally is present and inducible in wild type cells. ER from R3 elutes from DNA cellulose columns at a higher KCl concentration, and fails to show 'processing' although translocation occurs normally. The other variant clone, R27 is completely resistant to AE inhibition but interestingly retains E stimulation of both growth and PgR. ER from R27 is activated less effectively than in wild type cells and AE-ER complexes process abnormally. AE and E can induce synthesis of a secreted 46,000 MW glycoprotein in R27 whereas E stimulates and AE inhibits synthesis of this protein in wild type cells. We believe that both of these clones may represent receptor variants in which functions distal to the initial binding of hormone to receptor are altered.

0457 ANDROGEN RESISTANCE WITH DEFECTIVE NUCLEAR ANDROGEN BINDING, C. Eil*, D. Blank, & T.O. Fox, *Nat. Naval Med. Cen., Bethesda, MD 20814 and Harvard Med. Sch., Boston MA 02115

The androgen resistance (AR) syndromes are generally felt to be due to quantitative or qualitative abnormalities of the androgen receptor. Some patients with testicular feminization (TF) have no demonstrable cytosol androgen binding, while others have androgen binding which is thermo-sensitive or fails to be stabilized by Na molybdate. We describe here familial incomplete TF associated with reduced nuclear androgen retention. Fibroblasts, cultured from pubic skin biopsies of two phenotypic female XY siblings (age 20 and 18 yrs), were assayed for uptake of ^{3}H-dihydrotestosterone (DHT*). Binding was examined in dispersed, whole cells at 22°C and in nuclei extracted from intact cells incubated with DHT* at 37°C. DHT* binding in the patients' fibroblasts was normal by the whole cell method, while no high affinity, saturable nuclear binding of DHT* was demonstrable in the patients' cells at 37°C. Although cytosol from the patients' fibroblasts revealed a quantitatively diminished 8S peak for DHT* on Na molybdate-containing sucrose gradients, there was no peak of ^{3}H in the 4S region from nuclear extracts of the patients' cells which had been incubated with DHT* at 37°C. Finally, high salt extracts of the patients' cells incubated with DHT* at 37°C did not bind normally to DNA-cellulose. We conclude: 1) These data describe two cases of AR in which cytosol androgen receptors are present in fibroblasts but in which detectable nuclear DHT* uptake is impaired. 2) Assessment of nuclear DHT* uptake provides an effective indicator of the functional integrity of the androgen receptor system. 3) Altered androgen binding to DNA-cellulose of extracts from these patients' cells suggests that the molecular defect causing AR in these patients is in the soluble fraction.

0458 GLUCOCORTICOID AGONIST ACTIVITY OF DEXAMETHASONE-21-MESYLATE ON RPMI 3460-CLONE 6 MELANOMA CELLS, Alessandro Weisz[●], Robert L. Buzard[+], Diane Horn[+], Ming P. Li[○], Lois V. Dunkerton[○], and Francis S. Markland, Jr.[●]*, Department of *Biochemistry and [●]USC Comprehensive Cancer Center, University of Southern California, Los Angeles, CA 90033 and Department of [○]Chemistry and [+]Division of Molecular Biology, University of Southern California, Los Angeles, CA 90007

Dexamethasone-21-mesylate (DXM-M) is a glucocorticoid derivative able to interact covalently with the steroid binding site of the glucocorticoid receptor. Previous tests of the biological activity of this compound have shown a long-acting antiglucocorticoid effect at low concentrations as well as partial agonist activity at higher concentrations of the steroid derivative (Simons, S.S., and Thompson, E.B. Proc. Natl. Acad. Sci. USA 78, 3541-3545, 1981). These experiments were done with rat hepatoma (HTC) cells, a cell line in which glucocorticoids stimulate the expression of a specific enzyme, tyrosine aminotransferase.

We have studied the effect of DXM-M in RPMI 3460-clone 6 Syrian hamster melanoma cells, a cell line which is sensitive to growth inhibition by dexamethasone (DXM). Our results show that (1) DXM-M, like DXM, inhibits the growth of RPMI 3460-clone 6 cells, (2) although the dose-response curves are different, the magnitude of the biological response to DXM-M is at least as great as to DXM, (3) compared with DXM, the effect of DXM-M on these cells is less readily reversed upon removal of the steroid from the medium. Thus, DXM-M is a long-acting glucocorticoid agonist in these Syrian hamster melanoma cells. This result is in contrast with the observed long-acting antagonist (and partial agonist) activity of DXM-M in HTC cells.

0459 REGULATION OF GLUCOCORTICOID RECEPTOR ACTIVATION DURING THE CELL CYCLE. R. Alexander Currie and John A. Cidlowski, Dept. of Biochemistry, Univ. of Vermont College of Medicine, Burlington, VT 05405.

Glucocorticoids induce alkaline phosphatase activity (AP) during late G_1 and S phases of the HeLa S_3 cell cycle, but not during G_2, M, or early G_1 phases (Griffen and Ber, (1969) J. Cell Biol. 46, 297). In an attempt to understand why these cells appear refractory to glucocorticoid action during G_2, M, and early G_1 phases we have undertaken physicochemical analysis of the glucocorticoid receptor (GR). GR isolated from late G_1 and S phases eluted as a single predominant peak (Form I) from DEAE cellulose (DEAE) at 0.15 M KCl, and from hydroxylapatite (HAP) at 0.125 M K_2HPO_4. In contrast, GR isolated from G_2 and early G_1 phase cells eluted from both DEAE and HAP as a bi-phasic peak; a more highly acidic GR species (Form II) eluted from DEAE at 0.25 M KCl. A time course analysis of the apparent GR modification showed that initial receptor alteration began in late S phase, and reached a maximum in G_2 and Early G_1 phase. Correlation of altered receptor structure with impaired receptor function was demonstrated by the ability of GR to become "heat activated", and bind to DNA cellulose. During late G_2 and S the phases of the cell cycle GR binding to DNA cellulose were comparable. However, during G_2 and early G_1 GR binding to DNA cellulose decreased by 41 and 54% respectively. Analysis of total nuclear glucocorticoid binding in whole cells showed that nuclear receptor binding corrolated well to the ability to activate receptor *in vitro*. These data suggest that the glucocorticoid receptor may be a modifiable protein *in vivo* whose "activation" to the nuclear binding form is cell cycle dependent. Supported by NIH Grant AM 20892.

0460 GLUCOCORTICOID-CONTROL OF KERATINIZATION OF CHICK EMBRYONIC SKIN. INVOLVEMENT OF GLUCOCORTICOID RECEPTOR IN HYDROCORTISONE-INDUCED OR DMSO-INHIBITED EPIDERMAL CELL DIFFERENTIATION

Akiko Obinata, Momoko Kawada and Hiroyoshi Endo, Teikyo University, Kanagawa 199-01, Japan

Our morphological and biochemical studies have shown that hydrocortisone(10^{-8}M) induces keratinization of chick embryonic skin grown in a chemically defined medium. As dimethylsulfoxide (DMSO) affects differentiation of various cells in culture, we examined whether it affects chick embryonic epidermal cell differentiation. The presence of 2-4% DMSO with hydrocortisone reversibly prevented this keratinization. DMSO suppressed the synthesis and accumulation of epidermal structural protein and the increase of epidermal transglutaminase activity, which was presumably responsible for polymerization and decrease in solubility of epidermal protein in keratinization. Addition of a high concentration of hydrocortisone to the medium did not overcome the inhibition by DMSO, suggesting that DMSO does not compete with hydrocortisone at its action site. When added directly to the cytosol (105,000 g supernatant) of the skin homogenate, 2-4% DMSO did not inhibit the binding of ^{3}H-hydrocortisone to the cytosol. Higher receptor activity was found in the cytoplasm of the skin cultured with DMSO plus hydrocortisone when compaired with the skin cultured with the hormone alone. DMSO inhibited binding of the hormone to the epidermal nuclei. These findings suggest that in DMSO treated skin hydrocortisone -receptor complex can not translocate or bind to nuclei in epidermal cells, while the hormone can enter the cell and bind to its receptor.

Receptor Characterization

0461 MULTIPLE STATES OF NERVE GROWTH FACTOR RECEPTORS ON PC12 AND A875 CELLS: A COMPARISON OF STRUCTURE AND BINDING KINETICS, Stephen E. Buxser and Gary L. Johnson, Dept. of Biochemistry, University of Massachusetts Medical Center, Worcester, MA 01605

The kinetics of ^{125}I-labelled nerve growth factor (^{125}I-NGF) binding to specific receptors (NGFR) on PC12 rat pheochromocytoma and A875 human melanoma cells was evaluated. A reproducible increase in K_D for NGF from 0.38 nM to 3.5 nM with PC12 and 0.67 nM to 3.7 nM with A875 was observed when membranes were prepared from intact cells and isolated using sucrose density centrifugation. The change in K_D is a quantitative shift in affinity rather than a selective isolation of low affinity receptors. Specific photoaffinity crosslinking of the NGFR with ^{125}I-NGF using hydroxysuccinimydyl-p-azidobenzoate gives a M$_r$ 120,000 crosslinked peptide in both PC12 and A875 cells and membranes suggesting significant homology between receptors. The slow dissociating form of NGFR designated "SLOW-receptor" was found to be a property of the receptor which is independent of receptor affinity, and is present in both the high and low affinity forms of the NGFR. SLOW-receptor is present in PC12 cells and membranes but absent in A875 cells or membranes. The absence of SLOW-NGFR in A875 cells indicates a functional difference in this NGFR system compared to that in PC12 cells. PC12 membranes contain less SLOW-receptor as percent of total NGFR than intact PC12 cells, and NGFR solubilized from membranes fail to express the SLOW-form of receptor. These findings suggest that a single NGFR is present in multiple affinity states, and the NGFR may associate with other cellular components that induce either or both high affinity and slow dissociation of NGF. During membrane purification and solubilization of the NGFR these putative NGFR regulatory components appear to be lost or uncoupled.

0462 MODULATION OF THE NEURONAL BINDING OF THE β SUBUNIT OF NERVE GROWTH FACTOR(NGF) BY THE αNGF SUBUNIT Nathaniel R. Woodruff and Kenneth E. Neet Case Western Reserve University, Cleveland OH 44106

The effect of the α subunit of the 7S NGF on the binding of βNGF to its two classes of sites on target cells has been studied. The presence of μM concentrations of αNGF causes the displacement of ^{125}I-βNGF from one class of sites on dissociated dorsal root ganglia neurons from stage E9 chicken embryos. At 0.1nM ^{125}I-βNGF, increasing αNGF concentrations produce a monotonic displacement curve with half maximal displacement occurring at 10μM αNGF. The affinity and number of sites of the ^{125}I-βNGF displaced by αNGF are similar to those of βNGF that binds to the higher affinity (Site I) receptors. The binding to the lower affinity class of sites (Site II) is not affected by concentrations of αNGF up to 30μM. This modulation of ^{125}I-βNGF binding does not occur with equivalent concentrations of serum albumin. No detectable neuronal binding of ^{125}I-αNGF was found, suggesting that the mechanism does not involve direct competition for receptor sites. The dissociation constant for the α-β complex is in the μM range, therefore formation of this complex in solution can compete with the process of ^{125}I-βNGF binding to neurons. A model accounting for these observations includes binding of the α-β complex to the lower affinity but not to the higher affinity sites. We conclude that there are differences in the specificity of the two classes of receptors. (Supported by Am. Canc. Soc. Grant BC249)

0463 ALTERATIONS IN THE BINDING PROPERTIES OF NERVE GROWTH FACTOR RECEPTORS IN PC12 CELLS BY WHEAT GERM AGGLUTININ, Ronald D. Vale and Eric M. Shooter, Stanford University, Stanford, CA 94305

Nerve growth factor (NGF), a polypeptide hormone important in the development of the sympathetic nervous system, binds to a heterogeneous population of receptor sites on the rat pheochromocytoma cell line, PC12. To better understand the interactions of NGF with its receptor, we have studied how plant lectins affect NGF binding. Of eight lectins tested, only wheat germ agglutinin (WGA) caused a significant (50%) inhibition of binding when added 30 min prior to ^{125}I-NGF. Cells not treated with WGA have two types of NGF receptors which exhibit rapid (k_{-1} = 2 x 10^{-2} sec^{-1}) and slow (k_{-1} = 4 x 10^{-4} sec^{-1}) dissociation kinetics in the presence of excess unlabeled ligand. However, cells pretreated with WGA only demonstrate NGF binding to slowly dissociating receptors. If WGA is added after ^{125}I-NGF has reached equilibrium with cells, total binding remains unaltered, but the lectin converts all NGF bound to rapidly dissociating receptors into a slowly dissociating form. This conversion approaches a maximal response within 2 min at 37° or 4° C and occurs in the presence of metabolic energy inhibitors suggesting that the WGA-induced slowly dissociating receptors are not the product of internalization. Normally, most NGF binding is solubilized by 0.5% Triton X-100, a detergent which solubilizes membranes but leaves cytoskeletal structures intact. However, in the presence of WGA, NGF becomes associated with Triton inextractable cell material, indicating that the lectin may anchor the NGF-receptor complex to the cytoskeleton. The association of the NGF receptor with the cytoskeleton may be involved in the alteration of NGF binding properties induced by WGA.

0464 TUNICAMYCIN BLOCKS THE EMERGENCE OF INSULIN RECEPTORS ON HUMAN T LYMPHOCYTES, Thomas J. Brown, Louis Ercolani, and Barry H. Ginsberg, VAMC and DERC, Dept. of Internal Medicine, University of Iowa, Iowa City, Iowa, 52242.

Thymic derived peripheral human T lymphocytes do not bear insulin receptors. However, upon activation by alloantigens or mitogenic lectins insulin receptors are detectable on these cells within 24 hours. Since several lymphocyte cell surface receptors are known to be glycoproteins we tested whether tunicamycin (TM), an antibiotic known to inhibit intracytoplasmic glycosylation of proteins could influence the appearance of insulin receptors on phytohemagglutinin (PHA) activated T lymphocytes. TM > 0.1 μg/ml completely inhibited insulin receptor appearance on PHA treated T cells after 48 hours culture. ^{3}H - thymidine uptake in these cultures was totally inhibited as well. These effects could not be explained by loss of cell viability or number during culture. Cell cultures in which TM was removed displayed similar insulin binding capacity and ^{3}H-thymidine uptake as non-treated controls. However, N-acetyl-glucosamine, an inhibitor of TM, did not reverse TM's effects. TM did not effect ^{125}I-PHA binding to these cells or ^{3}H-α-aminoisobutyric acid uptake. However, TM did inhibit ^{3}H-leucine uptake into trichloroacetic acid precipitable protein by 50%. Although these data suggest glycosylation of proteins may be critical to the acquisition of insulin receptors by human T lymphocytes, other inhibitory effects of TM on biochemical events critical to lectin activation cannot be excluded to account for these findings.

0465 THERMAL BEHAVIOR OF THE INSULIN RECEPTOR, Stuart K. Calderwood, and George M. Hahn, Dept. of Radiology, Stanford University Medical School, Stanford, CA 94305

Investigation of the response of mammalian cells to heat stress constitutes both an intriguing research discipline and a new department of cancer therapeutics. The present study focusses on the effect of hyperthermia (42-45°C) on the insulin receptor in HA-1 Chinese hamster ovary cells in monolayer in vitro.

Heat caused a decrease in ^{125}I-insulin binding to cells which was both time and temperature dependent above 42°C. Scatchard analysis indicated that the decrease was due to numerical loss of receptors rather than decreased affinity for the hormone. Heating had no major effect on internalization of the hormone. Pretreatment of cells with mild hyperthermia produced a population of receptors which were highly resistant to subsequent heating. Whether this effect is due to elimination of a sensitive population of receptors by the pretreatment or due to induced resistance (thermotolerance) has not yet been uniquely determined.

The results indicate that thermal sensitivity of the insulin receptor parallels that of the cell as a whole. This may suggest a role as target site for hyperthermia either for the receptor per se or the plasma membrane in general.

0466 CHARACTERIZATION OF THE INSULIN RECEPTOR PURIFIED FROM RAT LIVER MEMBRANES, Gonul Velicelebi, Ramani Aiyer and Guido Guidotti, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA. 02138

We have purified the insulin receptor from rat liver membranes using an affinity column in which insulin was linked to agarose through a chemically cleavable bond. This was achieved by first reacting insulin with a bifunctional imidoester that has a sulfhydryl at the unreacted end and subsequently attaching the modified insulin to sulfhydryl agarose through a disulfide bond. Next, it was ascertained that this insulin derivative is capable of binding to insulin receptors and eliciting biological response in fat cells. The elution of insulin receptors bound to the affinity column was accomplished by reduction of the disulfide between insulin and agarose by 10 mM DTT, thus obviating the use of denaturing elution conditions that are otherwise needed to dissociate the receptor from insulin on the column. We have obtained the insulin receptor with significant purity (10-40%) and substantial yield (20%) via this approach. Insulin binding properties of the purified receptor resemble those observed with the solubilized receptor in crude extract, i.e., the binding data yield curvilinear Scatchard plots with comparable values of K_d for the two regions of the curves. SDS polyarylamide gel electrophoresis of the purified protein indicated the presence of three major bands with apparent molecular weights of 200, 150 and 50 K daltons. When ^{125}I-insulin was crosslinked to insulin receptors and analyzed by SDS-PAGE, it became apparent that the binding of ^{125}I-insulin to the 50K peptide is nonspecific, i.e., it cannot be competed off by native insulin while the binding to the 150 K peptide appears to be specific.

0467 CHARACTERIZATION OF HUMAN PLACENTA RECEPTORS FOR INSULIN AND BASIC SOMATOMEDIN, G.D. Armstrong, M.D. Hollenberg, B. Bhaumick and R.M. Bala, Dept. of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Alberta and Dept. of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan.

It has been found that the binding of insulin to basic somatomedin (SM) receptors and the binding of SM to insulin receptors parallels the SM-like effects of insulin and the insulin-like effects of SM on certain cultured cell lines. When insulin and SM receptors are solubilized from human placenta membranes they retain their ability to bind ligand. Preliminary characterization has shown that they are highly homologous structures. For example, gel filtration chromatography and affinity chromatography on wheat germ lectin-agarose suggests that the two receptors are glycoproteins with apparent molecular weights of 300,000. Moreover, photoaffinity labeling procedures followed by SDS polyacrylamide gel electrophoresis of β-mercaptoethanol-reduced samples demonstrated that both insulin and SM affinity-labeled at least one protein with a molecular weight of about 135,000. Upon electrophoresis, the non-reduced, photoaffinity-labeled receptors migrated as aggregates with molecular weights in excess of 300,000. In a separate set of experiments insulin and SM receptors in placenta membranes were affinity labeled using disuccinimidyl suberate (DSS) to crosslink ^{125}I-labeled ligand to receptors. This procedure and peptide mapping of the DSS-labeled receptors has demonstrated similarities and differences between the two. Methods are now available for the purification of both receptors by affinity chromatography on insulin-agarose or by immunoaffinity chromatography using sepharose-protein A. It is felt that peptide mapping of the purified receptors will help to elucidate further the elements that relate either to ligand binding or to cell activation.

0468 PARALLEL INDUCTION OF RECEPTORS FOR INSULIN AND INSULIN-LIKE GROWTH FACTOR-I IN DIFFERENTIATING 3T3-L1 MOUSE FIBROBLASTS, Joan Massagué and Michael P. Czech, University of Massachusetts Medical Center, Worcester, MA 01605

Covalent crosslinking of ^{125}I-labeled insulin, insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II) to membranes from several human and rodent cell types has allowed us to identify receptor structures for these three ligands. The high affinity insulin receptor is a disulfide-linked heterotetrameric complex (Mr=350,000) that minimally consists of two α subunits and two β subunits. This insulin receptor exhibits a high affinity for insulin and a lower affinity for IGF-I and IGF-II. The IGF-I receptor is a Mr=350,000 disulfide-linked complex with a subunit structure similar to the insulin receptor. The IGF-I receptor exhibits high affinity for IGF-I and a lower affinity for IGF-II and insulin. Affinity labeled IGF-II receptor consists of a Mr=250,000 polypeptide not disulfide-linked to other subunits, and exhibits high affinity for IGF-II, lower affinity for IGF-I and no affinity for insulin. These three classes of receptors are present on the cell surface of 3T3-L1 mouse fibroblasts. 3T3-L1 cells can be induced to differentiate into an "adipocyte" phenotype by treatment with insulin, dexamethasone and methyl-isobutylxanthine. When 3T3-L1 cells are induced to differentiate into adipocytes the number of IGF-II receptors in these cells remains unchanged but insulin receptors and IGF-I receptors undergo a marked and simultaneous increase in number under these conditions. The data indicate that the coordinate expression of the insulin receptor and the IGF-I receptor in differentiating 3T3-L1 cells is regulated by a common set of events.

0469 CONCANAVALIN A RECEPTOR-LINKED SIGNALLING SYSTEM IN LYMPHOCYTE PLASMA MEMBRANES: COMPARISON WITH THE INSULIN RECEPTOR-LINKED SIGNALLING SYSTEM IN ADIPOCYTE MEMBRANES, J. Seals, J. Beachy, T. Reardon, and M. Czech, U. Mass. Med. Ctr. Worcester, MA 01605

Binding of concanavalin A (Con A) to T lymphocytes elicits a mitogenic response and a range of metabolic effects. One of these effects is the stimulation of pyruvate oxidation and this effect is blocked by a protease inhibitor, tosyl arginine methyl ester. This response is similar to the response of adipocytes to insulin: 1) insulin elicits a similar range of metabolic effects, including increased pyruvate oxidation; 2) a proteolytic step has been suggested in the insulin signalling system; and 3) Con A binding to adipocytes elicits many insulin-like responses, probably via the same effector mechanism. We have used methods developed in studying the insulin effector system in adipocytes to study the Con A effector system in lymphocytes to further compare these responses. Incubation of lymphocyte plasma membranes (PM) with Con A, or adipocyte PM with insulin increased the release of an enzyme regulator which stimulates pyruvate dehydrogenase (PDH) when added to mitochondria from the same cell. In addition, the lymphocyte regulator stimulated PDH in adipocyte mitochondria. The release of stimulatory activity in both systems followed a similar time course, with activity reaching a peak in 2-5 min then decreasing with further incubation. The effect of insulin on adipocyte PM was mimicked by Con A or trypsin, and was inhibited by protease inhibitors. The effect of Con A on lymphocyte PM was mimicked by phytohemagglutinin but not by wheat germ agglutinin, a non-mitogenic lectin. These results suggest that the soluble regulators produced in these systems in response to their respective ligands may reflect a similar mode of transmembrane signalling.

0470 CHARACTERIZATION OF INSULIN-LIKE GROWTH FACTOR II-BINDING TO DETERGENT SOLUBILIZED RECEPTORS FROM RAT PLACENTA. James F. Perdue, Lady Davis Inst., Montreal, Canada and W.H. Daughaday, School of Medicine, Washington Univ., St. Louis, MO.

Studies by numerous investigators have established that IGF-I and -II interact with specific receptors on the surface of responsive cells and initiate metabolic events, i.e. DNA and cell replication which culminate in fetal and skeletal development. Since little is known of the cellular changes which occur subsequent to the interaction of IGFs with receptors, we have undertaken to isolate and characterize those from the rat placenta which bind IGF-II, exclusively. Placenta from 19-21 day old pregnant rats were removed, homogenized and an enriched plasma membrane fraction, R_3, isolated by differential centrifugation. R_3 was treated with 0.5 to 1% (w/v) n-octylglucoside in 50 mM MES buffer, pH 8.5 for 60 min at 24° or overnight at 0-4° and a soluble extract obtained following centrifugation at 100,000 x g for 60 min. This extract contained 40-60% of the protein present in the starting material and receptors which bound ^{125}I-IGF-II specifically, e.g. radioactive labelled and unlabelled IGF-II but not IGF-I or insulin bound to the solubilized receptors. Quantitation of IGF-II binding to the soluble receptor as a function of concentration established it to be similar to that of binding to the membrane associated receptor. Further molecular characterization of this receptor will be presented. Research supported by Grants to JFP from the Medical Research Council of Canada and the National Institutes of Health.

0471 ADIPOCYTE INSULIN BINDING SPECIES: THE SIZE AND SUB-UNIT COMPOSITION OF THE LARGER BINDING PROTEIN, H. Joseph Goren, C. Elliott, R.A. Dudley, University of Calgary, Calgary, Alberta, Canada, T2N 4N1.
Several investigators have reported that there are both large and small sized insulin binding proteins in plasma membranes; the larger protein demonstrates non-linear Scatchard binding while the smaller protein has linear binding. We now present evidence that the larger insulin binding species consists of 4 proteins of different sizes but each protein is composed of the same sub-units. Rat epididymal adipocyte plasma membranes were prebound with ^{125}I-insulin and then exposed to 1 mM disuccinimidyl suberate for 15 min at 2°C. The membranes were solubilized in 0.1% Triton X-100 and applied to a Sepharose 6B column. Peaks of radioactivity from the column were lyophilized, dialyzed, relyophilized, and analyzed by dodecyl sulphate gel electrophoresis (5%, 100/1;mono/bisacrylamide). Autoradiograms of the gels were scanned with a densitometer. The Sepharose chromatogram revealed 4 radioactive peaks: peak 1 at column void volume; peak 2, K_{av}=0.27; peak 3, K_{av}=0.77; and peak 4, K_{av}=1.09. Dodecyl sulphate electrophoresis of fractions in peak 2 demonstrated 4 bands on autoradiography; peak 1 did not enter the gel and peaks 3 and 4 ran with the dye front. Molecular weight estimates of the 4 insulin binding species in peak 2 were: 600K, 450K, 350K and 300K. Each of these species on dithiothreitol reduction yielded sub-units of M_r=135K, 45K and 18K. These results suggest that the large insulin binding protein in rat epididymal adipocytes contains several insulin binding species, and that these insulin binding species differ only in the number of sub-units and not the type of sub-units they contain. (Supported by CDA and MRCC grants.)

0472 COMPARISON OF INSULIN BINDING PROTEINS IN PLASMA AND NUCLEAR MEMBRANES OF OBESE AND LEAN MOUSE LIVER, Jo Alene Goidl, Medical Research Division, American Cyanamid Company, Lederle Laboratories, Pearl River, NY 10965
Insulin binding proteins associated with plasma membranes and nuclear membranes from obese (ob/ob) and lean (ob/+ and +/+) mouse liver were examined by electrophoresis and autoradiography after chemical crosslinking to [^{125}I]-insulin. A major insulin binding protein of M_r 120,000 and two less predominant proteins of M_r 90,000 and M_r 50,000 were observed in membrane preparations of each type. For all proteins the insulin binding was specific as determined by competition with unlabeled hormone. The relative order of insulin binding proteins in the different preparations decreased as follows: lean plasma membranes > obese plasma membranes > lean nuclear membranes > obese nuclear membranes. Contamination of the nuclear membrane fraction by plasma membranes was ruled out. Scatchard analyses of [^{125}I]-insulin crosslinked to plasma and nuclear membranes indicated that the decrease in hormone binding in the obese mouse is a result of a reduction in the absolute number of receptors. The findings presented in this study provide additional support for this conclusion by demonstrating that membranes from obese mice are comprised of the same set of apparently unaltered insulin binding proteins as membranes from lean mice. Further, the presence of similar insulin binding proteins in both nuclear and plasma membranes suggests a physiological relationship between these structures with respect to hormone binding and/or in the mechanism of action of insulin.

0473 RESOLUTION OF HIGH AND LOW AFFINITY EPIDERMAL GROWTH FACTOR RECEPTORS, C. King and P. Cuatrecasas, Wellcome Research Laboratories, Research Triangle Park, NC 27709
Receptors for the mitogen epidermal growth factor (EGF) have been implicated as mediators of cellular transformation and tumor promotion. 12-0-tetradecanoyl-phorbol-13-myristate (PMA) causes transient reductions in the internalization of EGF into human KB cells by decreasing the affinity of EGF receptors 7- to 12-fold. After 90 min., there is complete recovery of the high affinity apparatus. This transient decrease in binding capacity is caused by delaying the appearance at the plasma membrane of cryptic receptors of high affinity. Exposure of these high affinity binding sites is temperature dependent and is blocked by inhibitors of de novo protein synthesis. After short incubation periods (0 to 60 min.) with ^{125}I-EGF, up to 90% of the internalized ^{125}I-EGF can be rapidly dissociated from cells by the addition of PMA. This kinetically reveals a trypsin-insensitive, non-dissociating component of ^{125}I-EGF. With PMA, both the rate (maximal at 4 hr with PMA as compared to 24 hr) and extent (8-fold greater with PMA) of transfer of ^{125}I-EGF into this stable compartment is enhanced and accounts for a several-fold stimulation in ^{125}I-EGF accumulation at later times. Thus the internalization of ^{125}I-EGF can be resolved into two components, one into which EGF immediately partitions and is freely exchangeable with the extracellular medium and that is disrupted by PMA. A second and previously unknown stable compartment retains ^{125}I-EGF in the presence of PMA to a considerably greater extent than that of untreated cells. These results suggest that the accumulation of stable mitogen-receptor complexes may be an element involved in the control of cellular growth and tumor promotion.

0474 CHARACTERIZATION OF 165K AND 145K EGF RECEPTOR PROTEINS OF A431 CELLS, Cathleen R. Carlin and Barbara B. Knowles, The Wistar Institute, Philadelphia PA 19104

Antisera prepared by injecting syngeneic mice with human chromosome 7-containing man-mouse hybrids recognize the human receptor for EGF. Two receptor proteins, a 165K and a 145K component, are immunoprecipitated from A431 cells labeled with ^{125}I or inorganic ^{32}P. Both proteins are phosphorylated after immunoprecipitation of unlabeled material and incubation with (^{32}P-γ)ATP. Analysis of phosphoamino acids reveals that the receptor proteins are phosphorylated at tyrosine, serine, and threonine residues. The receptor proteins have been analyzed by two-dimensional electrophoresis and have apparent pI's of 7, although both display charge heterogeneity and have unique charge isomers. The peptide fragments of the two receptor proteins have been compared after immunoprecipitation and partial proteolysis with S. aureus V8 protease. The peptide fragments thus generated have been tested with regard to their ability to bind ^{125}I-EGF and to become phosphorylated after incubation with (^{32}P-γ)ATP. This work has been supported by NIH CA 8470 and T32 CA09171.

0475 THE INHIBITION BY EGF OF LH-RECEPTOR INDUCTION IN OVARIAN CELLS IS EXERTED AT A STEP DISTAL TO CYCLIC AMP, A. Nimrod and L. Rodgers, Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel.

In the maturing ovarian follicle, the granulosa cells (GC) become responsive to luteinizing hormone (LH) through acquisition of new LH-receptor sites. The process is induced by follicle stimulating hormone (FSH) and can be inhibited in vitro by EGF. We have studied the mechanism of this EGF effect in cultures of GC from immature hypophysectomized estrogen-treated rats. Exposure of GC for 48 h to EGF (5 µg/ml) did not inhibit a subsequent stimulation by FSH of cAMP production in the cell monolayers (0.20±0.02 vs. 0.28±0.02 nmole cAMP/dish/20 min; control vs. EGF treated), while the induction of LH-receptors by FSH was completely abolished. EGF was also effective in inhibiting LH-receptor induction by substances other than FSH, such as 8-bromo cyclic AMP (1 and 2 mM) and cholera toxin (20 µg/ml). Addition of isobutyl methyl xanthine, a phosphodiesterase inhibitor did not reverse the EGF effect on the induction by FSH. A lack of effect of EGF on phosphodiesterase was also found by direct measurement of the enzyme activity in homogenates of monolayers after 48 h of culture with the growth factor. It is concluded that the inhibitory effect of EGF is not exerted through reduction of cAMP levels in the cell since: (i) FSH-stimulable cAMP production is not impaired by EGF, (ii) addition of exogenous cAMP does not abolish the EGF effect, and (iii) EGF does not accelarate cAMP degradation.

0476 DO DIFFERENCES IN LH RECEPTOR CHARACTERISTICS MEDIATE GENETIC DIFFERENCES IN OVARIAN RESPONSE CHARACTERISTICS? Jimmy L. Spearow, R.E.P., C.H.G.D., Univ. of Michigan, Ann Arbor, Michigan , 48109

Previous studies in our lab have shown that genetic variation has a large effect upon ovarian gonadotropin response characteristics. The number of eggs ovulated by immature female mice in response to large doses of exogenous gonadotropins was 8±1, 52±3, and 56±4 for strains A, SJL, and C57BL/6, respectively. An analysis of inbred strains, F1's, and F2's of these strains revealed that the low strain differed from the high strains by one to two genetic loci, respectively. We next asked whether genetic differences in LH receptor characteristics mediated the above differences. The ovaries of immature females were removed 2 days after pregnant mares serum gonadotropin treatment, the time at which human chorionic gonadotropin (hCG) would normally be administered. The specific binding of ^{125}I-hCG per ovary X 10^{-3} was 223±14, 209±11, and 380±58 for lines A, SJL, and C57Bl/6, respectively. This implies that 1) the increased ovulation rate of SJL over A involves a genetic difference which is expressed subsequent to hormone binding and 2) the increased ovulation rate of C57Bl/6 over A involves a genetic difference in LH receptor number. The basal rate of cAMP production per ovary by ovarian pieces during a 3 hour culture period was similar among strains. However, the hCG stimulated rate of cAMP production was about 2.5 times higher both in lines SJL and C57Bl/6 than in A. These data suggest that the increased ovarian responsiveness in SJL is due to a genetic difference subsequent to hormone binding which involves the production of cAMP.

0477 USE OF A FLUORESCENT GnRH ANTAGONIST IN THE ANALYSIS AND PURIFICATION OF CELLS WITH GnRH BINDING SITES, Steven A. Edwards, Joseph Trotter, Jean Rivier and Wylie Vale, The Salk Institute, La Jolla, California 92037

The potent GnRH antagonist Ac-ΔPro^1,pF-D-Phe^2,D-Trp^3,D-Lys^6(N -TMR)-GnRH has been used in the flow cytometric analysis and purification by cell sorting of gonadotrophs from dispersed pituitary cells of female Sprague Dawley rats, aged 14-17 days. Cells binding the fluorescent ligand had a peak of fluorescent intensity 3.4 fold higher (arbitrary units) than the general pituitary population. Cells binding the ligand comprised 13.8% of the population (subtracting an autofluorescent background of 5.1%). Ligand binding could be inhibited with an excess of the non-fluorescent compound. The fluorescence labeled cells were larger than the general population as deduced by low angle light scatter and more granular as deduced by right angle light scatter. Sorting the cells on the basis of fluorescent intensity resulted in a five-fold purification of gonadotrophs relative to unsorted cells, and a 15-26-fold purification of gonadotrophs relative to negatively sorted cells, based on FSH and LH radioimmunoassay of cell content. The present study illustrates the utility of fluorescent GnRH antagonists in the study of gonadotrophs. The general method could presumably be extended to the study of other cells containing GnRH binding sites in ovary, testes, and brain.

0478 INTERACTION OF CYCLIC NUCLEOTIDES WITH THE GONADOTROPIN-RELEASING HORMONE RECEPTOR ON PITUITARY AND OVARY, Mark A. Smith, Marilyn H. Perrin and Wylie Vale, Peptide Biology Laboratory, The Salk Institute, La Jolla, CA 92037

Studies described here suggest that exogenous cyclic nucleotides may interact directly and specifically with GnRH membrane receptors. Millimolar concentrations of 8-Br cAMP, dibutyryl cAMP and cyclic AMP produced significant LH and FSH release from cultured rat anterior pituitary cells while 5'-AMP and 2-chloroadenosine were without effect. However, the addition of a potent GnRH antagonist (10 nM [AcΔ^3Pro^1,pFD-Phe^2,D-Trp^{3,6}]-GnRH) completely blocked the stimulatory effects of these cyclic AMP derivatives but had no effect on LH secretion induced by the calcium ionophore A23187. By incubating pituitary or ovary membrane homogenates at 0° with tracer concentrations of the GnRH agonist [^{125}I-Tyr^5,D-Ala^6,N$^{\alpha}$MeLeu^7,Pro^9-NEt]-GnRH and increasing concentrations of peptide or cyclic nucleotides, we found that various cAMP analogs (ED_{50}'s 1-6 mM) were able to inhibit binding of radioligand to the same extent as the GnRH agonist. Computer analysis of GnRH agonist binding in the presence of 2 mM 8-Br cAMP revealed that the apparent affinity was reduced while the number of high affinity sites was not changed suggesting that the cyclic nucleotides act in a competitive manner to inhibit GnRH binding. Cyclic GMP analogs also inhibited GnRH binding, but in contrast to the stimulatory effects of cAMP analogs, they inhibited GnRH-stimulated LH release. Based upon slime mold studies, cyclic nucleotides have been proposed as prototype extracellular regulatory messengers. The interaction of cyclic nucleotides with the GnRH receptor may reflect the retention of ancient properties by some, but not all, peptide receptors.

0479 REGULATION OF Ca^{++} ACTION POTENTIALS BY THE TRH RECEPTOR. R. L. Vandlen, G. Kaczorowski, J. Reuben$^+$, G. Katz$^+$, A. Eastwood$^+$, Merck Institute for Therapeutic Research, Rahway, NJ and $^+$Dept. of Neurology, Columbia University, College of Physicians and Surgeons, New York, NY.

Secretion of hormones from pituitary cells requires the presence of extracellular Ca^{++}, the entry of which is controlled by voltage sensitive Ca^{++} channels in the plasma membrane. The duration of Ca^{++} action potentials is regulated by the opening of K^+ channels to repolarize the cell and thereby inhibit Ca^{++} channels. Thyrotropin releasing hormone (TRH) stimulates the secretion of prolactin from a pituitary tumor cell line (GH_3) after interaction with its receptor by apparently increasing the frequency and duration of these action potentials, thereby allowing increased intracellular levels of Ca^{++}. We have further investigated these responses by electrophysiological studies in both the parent GH_3 line and a new, morphologically distinct, clonal line (XG-10) whose cells are not responsive to TRH and are completely devoid of these receptors. Additionally, these cells have substantially increased basal and stimulated secretion rates compared to the parent cells. Intracellular recordings with the XG-10 cells have shown that their Ca^{++} action potentials were of substantially longer duration than those of the parent cells (500 to 2000 msec as compared to 10 to 50 msecs). The addition of the potassium channel blocker tetraethylammonium elongated the action potentials in the GH_3 cells but had no effect in XG-10 cells. These and other results suggest that one difference in the XG-10 cells is the loss of some or all of the voltage dependent K^+ channels. Since other experimental results have suggested that TRH modulates K^+ fluxes directly in pituitary cells, it is proposed that the TRH receptor may regulate or be part of the K^+ channel in pituitary cells.

0480 CHARACTERIZATION OF THE HOST CELL RECEPTOR FOR MURINE LEUKEMIA VIRUS, Marsha R. Rosner, Pegram A. Johnson and Christine A. Kozak*, Massachusetts Institute of Technology, Cambridge, MA 02139 and the National Institutes of Health*, Bethesda, MD 20014

The entry of retroviruses into host cells is mediated by a specific interaction between the envelope glycoprotein and a host cell receptor. In order to determine how specific leukemia viruses are recognized by their respective host cells, the cell surface receptor for murine leukemia virus is being characterized. The strategy is to to use Chinese hamster-mouse somatic cell hybrids as the host cell system, doing parallel studies on cells which contain or lack mouse chromosome 5 which codes for the murine leukemia virus receptor. The isolated envelope glycoprotein, gp70, from Moloney leukemia virus, is used as the ligand for binding studies.

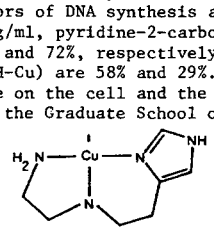
Initially a somatic cell hybrid (BM3C) was isolated which contains mouse chromosome 5 but lacks at least 11 of the remaining 19 mouse chromosomes. BM3C can be infected by the ecotropic Moloney virus, but not the Moloney recombinant (HIX) or amphotropic viruses. Incubation of the hybrid cell line BM3C with varying concentrations of 125I-labelled gp70 indicated that binding to receptor-containing cells is specific, saturable, and comparable to that observed with the mouse fibroblast line SC-1. No specific binding was observed with the Chinese hamster fibroblast line E36. Studies of the bound gp70 following mild detergent extraction have suggested that the viral receptor is associated with the detergent-insoluable cytoskeletal matrix of the host cell. These results indicate that the somatic cell hybrids provide an excellent system for probing viral receptor interactions against a common genetic background.

0481 PRECLUSTERING OF THROMBIN RECEPTORS ON MOUSE EMBRYO FIBROBLASTS, ANALYZED BY IMMUNOFLUORESCENCE, IMMUNOHISTOCHEMISTRY AND EM AUTORADIOGRAPHY, John S. Bergmann and Darrell H. Carney, Univ. of Texas Medical Branch, Galveston, TX 77550

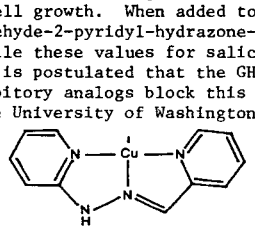
Thrombin interaction with receptors on the surface of fibroblasts appears necessary for thrombin to initiate cell proliferation. To visualize this interaction, we have used indirect immunofluorescence techniques with affinity purified thrombin antibody. These studies have shown that thrombin appears to bind preclustered receptors on the surface of mouse embryo and CHO cells incubated at 4° or prefixed with 3% formaldehyde (J. Cell Biol. 91, 230a). Additional studies with thrombin antibody now show that cells prefixed in up to 1% glutaraldehyde show the same preclustering, and that thrombin binding to these clusters correlates with high affinity receptor binding and is not an association with protease nexin. EM autoradiography studies confirm preclustered thrombin receptors. After binding ^{125}I-thrombin at 4°, 37° or to prefixed cells, the autoradiographic grains on cell surfaces were uniformly distributed with even spacing between grains. The distance between these grains corresponds to the distance between clusters of thrombin observed on these cells with immunofluorescence. Quantitation of grain number per location and number of thrombin molecules represented by each autoradiographic grain indicate that at 125ng/ml about 360 thrombin molecules are bound to each cluster. In these studies we found no vesicles or coated pit regions of the membrane with large numbers of autoradiographic grains. These results suggest that the occupied receptor clusters do not redistribute into larger aggregates prior to processing or endocytosis. (Supported by NIH Grant AM-25807).

0482 GROWTH-INHIBITORY ANALOGS OF THE GLY-HIS-LYS-CU(II) COMPLEX. Loren Pickart*,William H. Goodwin*, Terrance B. Murphy+ and David K. Johnson+. Virginia Mason Research Center*, Seattle, WA 98101 and University of Washington+, Seattle, WA 98195.

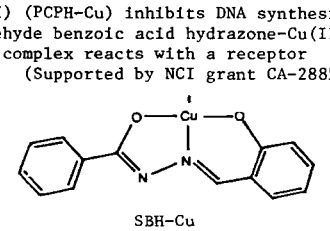
The growth factor, Gly-His-Lys (GHL) is an avid binder of copper and is postulated to function as a chelate of copper II (GHL-Cu) to alter the growth rate or state of differentiation of a wide variety of cultured cells and organisms. Structural studies (x-ray, e.p.r., spin echo, computer modeling) of GHL-Cu have delineated the 3-dimensional structure of the complex in solution (In Vitro 17: 459 (1981)). Copper is bound by GHL to form a three-ringed planar triaza tridentate complex. Certain synthetic analogs of the copper-binding region are potent inhibitors of DNA synthesis and cell growth. When added to human fibroblast cultures at 10 and 1 ng/ml, pyridine-2-carboxaldehyde-2-pyridyl-hydrazone-Cu(II) (PCPH-Cu) inhibits DNA synthesis 84% and 72%, respectively, while these values for salicyaldehyde benzoic acid hydrazone-Cu(II) (SBH-Cu) are 58% and 29%. It is postulated that the GHL-Cu complex reacts with a receptor site on the cell and the inhibitory analogs block this site. (Supported by NCI grant CA-28858 and the Graduate School of the University of Washington.)



GHL-Cu binding region



PCPH-Cu



SBH-Cu

0483 HETEROGENEITY OF HUMAN T LYMPHOCYTES SHOWN BY LABELING OF WHEAT GERM AGGLUTININ BINDING OF TRANSMEMBRANE GLYPROTEINS, Pedro Pinto da Silva and Maria Rosaria Torrisi, Section of Membrane Biology, Laboratory of Pathophysiology, National Cancer Institute, NIH, Bethesda, MD 20205.

We have recently described "fracture-label" techniques that permit direct cytochemical labelling of freeze-fractured cells. We report here the use of "fracture-label" to investigate the distribution and partition of wheat germ agglutinin (WGA) receptor sites over the protoplasmic and exoplasmic plasma membrane faces of freeze-fractured human T lymphocytes. All exoplasmic faces are strongly labelled by WGA. In contrast, over the protoplasmic faces label exhibits remarkable variation, ranging from virtual absence of label in some faces to very high densities in other faces. We interpret the presence of WGA receptor sites over the protoplasmic faces to reflect the presence of transmembrane WGA binding sialoglycoproteins that, during freeze fracture, partition with the inner half of the plasma membrane. Therefore, our results indicate heterogeneous expression of integral membrane proteins within populations of human T cells. Fracture-label techniques may represent an additional tool in the definition of lymphocyte subpopulations.

0484 REGULATION OF ACETYLCHOLINE RECEPTOR ACTIVITY IN THE ADRENAL MEDULLA, Oren Zinder and Avital Greenberg, Rambam Medical Center, Haifa, ISRAEL

Acetylcholine (ACH) induces release of catecholamines (CAT) from cells of the adrenal medulla. The kinetics of secretion were found to be temperature dependent with maximal secretion at 30°C. Temperature dependency of the initial rate had no maximum and increased with rising temperatures up to 44°C. The total amount of CAT released to the medium had biphasic kintics showing an increase up to a maximum at 30°C and then a decrease with further temperature rise. On the other hand, secretion initiated by 65mM K^+ or the calcium ionophore A23187, showed a monotonic increase with temperature rise having three distinct regions of secretion rate. The first was in the range of 4-15°C, the second was between 16-30°C and the third was at temperatures over 30°C. The decrease in secretion in the presence of ACH at temperatures over 30°C points to a possible desensitization mechanism for the receptor. This could be due to translocation or masking of the receptor within the medulla cell membrane, a process not invoked by high K^+ or A23187. In studies on the tensile strength of the secretory granule membrane, a transition at 16-19°C was seen. The transitions seen in the three regions of release rate in whole cells and in that found in the isolated granules, indicate possible involvement of phase transitions in the two membranes and support the hypothesis that the regulatory control of CAT secretion from the adrenal medulla is dependent on membrane fluidity facilitating fusion and secretion while at the same time allowing only a short exposure of the ACH receptor to take place. The result of this fine-control mechanism is a measured secretion of CAT for every stimulated release event.

0485 HUMAN TRANSFERRIN RECEPTOR: EXPRESSION OF THE RECEPTOR IS ASSIGNED TO CHROMOSOME 3, Caroline A. Enns, Heli A. Suomalainen, Janice E. Gebhardt, Jim Schröder and Howard H. Sussman, Stanford University School of Medicine, Stanford, CA 94305 and Folkhälsan Institute of Genetics, Helsinki, Finland

Human chromosome 3 has been identified as being responsible for the expression of the transferrin receptor in mouse/human lymphocyte hybrids. The receptor was detected by immunoprecipitation with anti-human receptor antibody of ^{125}I-cell surface labeled cells. This method also detected a similar 94,000 dalton protein in mouse cells. A radioimmunoassay developed for the human transferrin receptor measured 10% crossreactivity with the mouse protein. The two proteins were distinguished by sodium dodecyl sulfate-polyacrylamide gel patterns of partial proteolytic digests of the immunoprecipitated proteins.

Mouse/human hybrids were generated by fusing a mouse thymoma (BW5147) cell line to either Concanavalin A or pokeweed mitogen-activated human peripheral blood lymphocytes. Each hybrid was karyotyped with respect to both mouse and human chromosomes. In every case the expression of the human transferrin receptor correlated only with human chromosome 3.

0486 "NEXINS:" A FAMILY OF CELL-SECRETED PROTEINS THAT SELECTIVELY MEDIATE THE SPECIFIC CELLULAR BINDING OF REGULATORY SERINE PROTEASES, Daniel J. Knauer and Dennis D. Cunningham, University of California, Irvine, CA 92717

Epidermal growth factor carrier protein (EGF-CP) and the subunit of 7S nerve growth factor (NGF) are serine proteases that proteolytically process PRO-EGF and PRO-NGF, respectively, to the commonly isolated forms of EGF and NGF-β. In addition, both EGF-CP and NGF-γ directly enhance the biological activity of EGF and NGFβ, respectively. To probe the biochemical basis of this enhancement, we investigated the interactions of EGF-CP and NGF-γ with cultured human fibroblasts (HF cells). We identified two unique cell-secreted proteins that mediate the specific binding of EGF-CP and NGF-γ to HF cells. We named these proteins EGF-CP nexin ($M_r \sim 95K$) and NGF-γ nexin ($M_r \sim 28K$) because of their close functional similarities to protease nexin that mediates the cellular binding of thrombin and urokinase (1,2). Using ^{125}I-EGF-CP and ^{125}I-NGF-γ we demonstrated the formation of ^{125}I-EGF-CP:nexin and NGF-γ:nexin complexes in serum-free HF-cell conditioned medium by SDS gel electrophoresis, and subsequently verified that these complexes account for the cellular binding and internalization of EGF-CP and NGF-γ. These studies, in conjunction with data from competitive binding experiments enabled us to conclude that EGF-CP nexin and NGF-γ nexin are unique proteins that are distinct from protease nexin. Taken together, these data suggest that cell secreted "nexins" represent a family of proteins that provide a novel and highly specific mechanism for the interaction of cells with regulatory proteases.
1. Cell 21:37-45 (1980). 2. Proc. Natl. Acad. Sci. USA 78:2334-2340 (1981).

0487 EVOLUTION OF RECEPTORS IN BACTERIAL CHEMOTAXIS, Dennis O. Clegg, Elizabeth A. Wang, Andrew F. Russo and Daniel E. Koshland, Jr., University of California, Berkeley, CA 94720

The gene for the aspartate receptor involved in bacterial chemotaxis is shown to be tandemly duplicated. The two genes, designated tarA and tarB, have been separated on recombinant plasmids and analyzed. The genes function identically in complementation tests but differences in DNA sequence homology have been detected by Southern blotting. Both genes encode 60K dalton integral membrane proteins that are multiply methylated and mediate taxis to the attractants aspartate and maltose and the repellents Ni^{2+} and Co^{2+}. It has previously been shown that the tar gene product is the primary receptor for aspartate and appears to interact with the periplasmic maltose binding protein to transduce maltose responses. The methylation reaction has been shown to be responsible for sensory adaptation in this system.

Interestingly, this duplication occurs in E. coli but not in the closely related species Salmonella typhimurium. The selective advantage that has maintained the duplicated receptor gene is unknown, but the second copy may have diverged to allow taxis towards additional effectors. A number of other cross-hybridizing sequences are found in the genomes of both species and their homologies have been quantified. The receptor genes appear to have duplicated and diverged to expand the range of environmental stimuli while maintaining enough homology for the proteins to be methylated by the same modifying enzymes.

0488 CHARACTERIZATION AND COMPARISON OF THE CHEMOTACTIC RECEPTORS OF D. DISCOIDEUM, Beth L. Meyers and William A. Frazier, Washington University School of Medicine, St. Louis, MO 63110

As D. discoideum cells differentiate their chemotactic system changes its specificity from folic acid in the vegetative state to cAMP during the aggregation phase. Folate and cAMP receptors are present during development and either can be coupled to the chemotactic machinery indicating they may share a common transducer mechanism. These receptors are integral membrane proteins with extremely rapid dissociation rates. Until recently this characteristic hindered study of these proteins in the solubilized state. Development of a hydrophobic immobilization assay has enabled us to partially purify and characterize the cAMP receptor which has been solubilized with Emulphogene BC-720. In its crude state, the solubilized cAMP receptor has the same binding characteristics as those of the receptor in plasma membranes, including its affinity for cAMP versus other nucleotides and inactivation by sulfonyl fluorides, features which distinguish it from the solubilized membrane phosphodiesterase. Partial purification of the cAMP receptor has been achieved using chromatography on DEAE-Sephadex and decyl agarose. Photoaffinity labelling studies with 8-N_3-γ-^{32}P-cAMP indicate that a protein of M_r 70,000 is responsible for the cAMP binding activity. This partially purified cAMP receptor shows the same binding characteristics as that in membranes or intact cells. Using [^{3}H]methotrexate as a ligand, studies of the folate receptor in plasma membranes and the detergent-solubilized state are being carried out. Comparative studies of the folate and cAMP receptors may provide clues regarding the mechanism of signal transduction. [Supported by NS 13269 and PCM-78 04304. WAF is an Established Investigator of the American Heart Assn.]

0489 IDENTIFICATION OF THE CHEMOTACTIC RECEPTOR OF D .DISCOIDEUM AND ITS MODIFICATION UPON DOWN-REGULATION. Claudette Klein and Jennifer Lubs-Haukeness. St. Louis University Medical School, St. Louis, MO. 63104

D. discoideum amoebae respond chemotactically to external pulses of cAMP. The effects of external cAMP are mediated by specific cell surface receptors. One of the first events in the translation of the external signal into a locomotory response is a transient activation of adenylate cyclase and the rapid excretion of the newly synthesized cAMP into the medium. Subsequent to stimulation with cAMP, amoebae become temporarily desensitized to further stimulation. It is not clear if transitory changes in cell sensitivity to cAMP relfect altered cAMP binding to the cell surface. External cAMP can elicit a down-regulation of its receptors. This phenomenon appears to be correlated with the formation of a cAMP binding component from which cAMP slowly dissociates. Using the photoaffinity analogue, N_3cAMP, we have identified, by all available criteria, a 45,000 molecular weight protein (p45) as the cell surface cAMP receptor. When cells are treated with cAMP to down-regulate their receptor sites by 70%, this component is no longer labeled: instead a protein of 47,000 molecular weight is now present. This cAMP-induced form of the cAMP receptor may represent a new binding protein or a modification of p45. Our recent experiments suggest that, if the latter is true, phosphorylation my be involved. Conditions which cause receptor down-regulation and the appearance of p47 also result in the specific phosphorylation of a 47,000 molecular weight protein (pP47). Both the concentration dependence and time course of p47 and pP47 induction by cAMP appear coincidental. Work is in progress to discern if cAMP does effect the phosphorylation of its receptor and the role of such an event in regulating receptor activity or its coupling to adenylate cyclase.

0490 IDENTIFICATION AND LOCALIZATION OF SPECIFIC SURFACE SITES ON THE REGULATORY SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE USING MONOCLONAL ANTIBODIES AND PHOTOAFFINITY LABELING. S.L. Weldon, A.R. Kerlavage and S.S. Taylor; U. Calif. San Diego, La Jolla, CA 92093.

The regulatory subunit (R) of type II cAMP-dependent protein kinase has several functional sites including those for cAMP binding, autophosphorylation, and recognition of the catalytic subunit. The photoaffinity analog 8-N_3cAMP has been used to directly distinguish the 2 cAMP-binding sites on each R monomer. 8-N_3cAMP stoichiometrically modifies a single tyrosine residue at one of the cAMP-binding sites. This site has been sequenced and localized at the C-terminal end of the polypeptide chain. Millipore filtration assays under varying conditions established that holoenzyme (R_2C_2) binds 4 mol/mol cAMP whereas only 2 mol 8-N_3cAMP bind per mol R_2C_2. Since cAMP and 8-N_3cAMP are equally capable of activating and dissociating holoenzyme, it is clear that occupation of a single cAMP-binding site, specifically that site recognized by 8-N_3cAMP, is sufficient to activate the cAMP-dependent holoenzyme.

Monoclonal antibody has been used to further probe surface sites on the regulatory subunit. Using limited proteolysis and CNBr cleavage, a specific region of the linear polypeptide chain has been characterized that retains the antigenic site. Subsequent cleavage of the immunoreactive CNBr peptide with chymotrypsin has further localized the antigenic site to a 20-residue sequence that is in close proximity to the site of autophosphorylation. Since this region is also important for recognition of the catalytic subunit, for cAMP binding, and may contain a histone-binding site, this antibody may be useful for understanding functional relationships associated with this 30-residue region of the molecule.

(Supported by USPHS Grant GM 19301 and NSF Grant PCM 78-10155).

Comparison of Steroid and Polypeptide Hormone Mechanisms of Gene Regulation

0491 EVIDENCE FOR THE EXISTENCE OF A PROLACTIN SECOND MESSANGER, P.A. Kelly, J. Djiane, L.M. Houbedine and B. Teyssot, CHUL, Quebec G1V 4G2, and INRA, 78350, Jouy-en-Josas.

An intracellular relay for prolactin was studied using isolated rabbit mammary nuclei incubated in the presence of Hg CTP. The neosynthetized mecurated RNAs were isolated with SH-Sepharose columns and their content in β casein mRNA sequences was estimated using a labelled β-casein cDNA probe. A soluable factor was released following the incubation of prolactin or other lactogenic hormones with rabbit mammary gland microsomes. The membrane supernatant specifically stimulated the transcription of β-casein genes 5 to 10 fold. The amount of stimulation was dependent on the amount of supernatant protein added to the nuclei (optimum 200 μg) and on the concentration of prolactin added to the membranes (optimum 1000 ng/ml). The active factor was not generated by incubation of membranes at 4°C and appeared faster at 37°C than 20°C. The mediator retained its activity after heating at 100°C for 10 min but was inactivated by high concentrations of trypsin. As judged by chromatography on Sephadex G-25, the mediator had a molecular weight of 1000-1500 daltons. An anti-prolactin receptor antibody, which mimics the action of prolactin in the mammary gland, also released this mediator. Colchicine, which blocks the action of prolactin in the mammary gland, prevents the production of the mediator. This soluble factor can be produced by other tissues containing prolactin receptors, such as rat liver. Interestingly, plasma membrane fractions were very active, whereas Golgi membranes, which contain approximately 10-fold more receptors, produced very little mediator. These data suggest that the binding of prolactin to its receptors on the plasma membrane induces the formation of an intracellular relay with some characteristics of a polypeptide, which specifically stimulates the transcription of prolactin-sensitive genes.

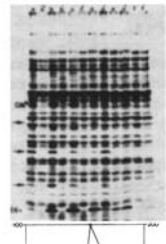
0492 NUCLEAR UPTAKE OF THE INDUCER Ah RECEPTOR COMPLEX IN MOUSE LIVER, Howard J. Eisen, Rita R. Hannah, Robert H. Tukey, Masahiko Negishi and Daniel W. Nebert, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20205

The Ah locus is associated with the induction of certain drug-metabolizing enzymes by polycyclic aromatic compounds such as 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)]. The major regulatory gene product is the cytosolic Ah receptor, which has molecular weight ~250 kd and binds avidly to TCDD (apparent K_d ~0.5 nM). One of the inducible structural gene products is cytochrome P_1-450, the monooxygenase that best metabolizes chemical carcinogens such as benzo[a]pyrene. When mice are treated with [^{3}H]TCDD *in vivo*, the [^{3}H]TCDD-Ah receptor (T-R) complex accumulates in the nucleus. The T-R complex is detectable in both cytosol and nucleus of "Ah-responsive" mice but is found in small amounts only in the nucleus of "Ah-nonresponsive" mice. With the use of a cloned portion of the P_1-450 structural gene, P_1-450 mRNA induction is measurable with as few as 200 T-R molecules in the nucleus. The cytosolic receptor (ligand-free) and T-R complex do not bind to DNA-cellulose, whereas the nuclear T-R complex binds to DNA-cellulose (but not cellulose alone) and is eluted as a single peak (0.38 M) during a NaCl gradient. The elution profile is identical for double- and single-stranded DNA-celluloses. Heat treatment (25° C, 1 h), dilution, or exposure to increased ionic strength (1 M NaCl) does not "activate" the cytosolic T-R complex, yet any of these treatments activates the cytosolic glucocorticoid-receptor complex in mouse liver. Hence, the cytosolic and nuclear forms of the T-R complex differ in their affinity for DNA, and the T-R complex differs in some ways from the glucocorticoid-receptor complex.

0493 THE EGF RECEPTOR OF THE BeWo CHORIOCARCINOMA CELL LINE. K.A. Valentine and M.D. Hollenberg, Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada.

The BeWo choriocarcinoma cell line exhibits two distinct phenotypes under normal culture conditions. The majority of the cells (99%) are small, mononucleated and proliferative bearing a morphological resemblance to the *in utero* cytotrophoblast (cytotrophoblast-like or CTL cells). The remaining 1% of the culture is composed of large, multinucleated, nonproliferative cells which are syncitiotrophoblast-like in morphology (STL cells). Treatment of BeWo cultures with 1 μM methotrexate (MTX) results in a transformation of the culture phenotype to one that is predominantly STL in morphology. The MTX-STL cells are morphologically indistinguishable from the naturally occurring STL cells. Both CTL and MTX-STL cells have been found to possess membrane receptors for epidermal growth factor (EGF). EGF binding is saturable at 24° and 4°C. Scatchard analysis of the binding data for the CTL-EGF receptor shows that these cells possess approximately 1.8×10^5 binding sites/cell with an affinity constant (K_d) of 2.5×10^{-9} M. CTL cells are also capable of down-regulating their EGF receptors upon exposure to the hormone at 37°C. Preliminary binding studies using MTX-STL cells demonstrate that the change in cell morphology is accompanied by an increase both in the affinity of the EGF receptor and in the number of binding sites available per cell. Affinity labelling and electrophoretic analysis of the CTL-EGF receptor indicates that the receptor has a molecular weight comparable to that seen for the human placenta membrane EGF receptor. The BeWo cell line therefore, may provide a useful model for the study of receptor changes in a differentiating system.

0494 CELL MEMBRANE RECEPTORS REGULATE CELLULAR COMPETENCE IN STREPTOCOCCUS SANGUIS. J.L. Raina, University of Chicago, Chicago, Illinois 60637



Streptococcus sanguis cell surface receptor(s), after interaction with the ligand, impart information that transiently modifies cellular physiology. This transient change, called competence, is defined by the synthesis of a set of new polypeptides(14K to 51K,Fig.1,→,Δ) and characterized by the uptake and expression of exogenous donor DNA by recipient cells. Two inducible polypeptides, a 16K donor single-stranded DNA binding protein(E16) and a 51K recombination-specific protein(cipA), have already been implicated in this transformation process. Procaine and tetracaine inhibit transformation selectively by preventing the expression of inducible polypeptides, specifically during early onset of competence development(Fig.1,a'-c'). The reversible effect of procaine appears to be mediated by cell membrane receptors elaborated or unmasked before the onset of competence. Post competence phase cells lose their affinity for binding ^{14}C-labeled procaine and are consequently unaffected by its inhibitory effects(Fig.2). Methyltriphenyl phosphonium bromide, a strong base cation known to alter membrane potential, appears to inhibit transformation selectively by preventing the processing of inducible E16 polypeptide precursor to mature proteins. These studies provide the first evidence for a relationship between cellular membrane potential (Δψ) and energy linked changes in hydrophobic membranes in regulating cellular competence for genetic transformation in bacteria.

Evolution of Hormone-Receptor Systems

Application of Recombinant DNA Techniques to Studies of Hormone Receptor Evolution and Action

0495 HUMAN GROWTH HORMONE: A MULTIGENE FAMILY David D. Moore, Mark A. Conkling, and Howard M. Goodman, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

Human growth hormone (hGH) is a polypeptide hormone which is encoded by a member of a highly homologous multigene family. The closely related placental hormone chorionic somatomammotropin (hCS) is the only other protein known to be expressed by this family of genes. We have isolated at least six distinct members of the hGH family from the DNA of two individuals. Characterization of these sequences by quantitative hybridization and sequence analyses shows that hGH and hCS are the most disparate members of this family. Two of the other members are extremely homologous variants of the hGH and hCS genes. This multigene family also includes an hGH/hCS hybrid in which the first exon is hGH-like and at least the next three are identical to hCS.

In addition to this potential diversity at the level of the genome, the hGH family also exhibits diversity at the level of RNA processing. In normal pituitary cells there are two alternative pathways for splicing the hGH mRNA precursor. These two splicing patterns generate distinct mRNAs that encode hGH polypeptide which differ by an internal deletion of 15 amino acids.

0496 Abstract withdrawn

Comparison, Contrasts, and Interactions among Different Protein Phosphorylating Systems

0497 COMPARATIVE STUDIES OF PROTEIN KINASE SYSTEM IN NORMAL AND NEOPLASTIC PROSTATIC TISSUES, Leland W.K. Chung and Karen Breitweiser, Univ. of Colorado, Boulder, CO 80309
Protein kinase isoenzymes were determined in prostatic cytosol isolated from normal prostates and prostatic tumors (Dunning tumors, hormone-dependent {HDT}, hormone-independent {HIT}, and anaplastic {AT}). Three major regulatory subunits of protein kinase isoenzymes, Mr. 58,000, 51,000 and 42,000 were detected in prostatic cytosol: they represent the regulatory subunits of type II (R_{II}), type I (R_I) and proteolytic fragments (R_{pf}) of protein kinases, respectively. Marked quantitative differences as determined by photoaffinity labeling with 8-N_3-(^{32}P)-cAMP were observed in R_I, R_{II} and R_{pf} between normal prostates and Dunning tumors. HDT and HIT or AT differed from the normal prostates and can be differentiated among them based on their relative binding to 8-N_3-cAMP. In addition, the binding kinetics to 8-N_3-cAMP for R_I was inhibitory in HIT and AT but saturation was observed in normal prostates and HDT. The ontogeny and androgen responsiveness of the cytosolic protein kinase system were also studied in Noble (Nb) rats. Results indicate that only the type II protein kinase activity (histone was used as a substrate) eluted from a DEAE-cellulose column, was greatly decreased as a function of age. Long-term testosterone propionate administration as Silastic implants completely depleted both types of cytosolic protein kinases. This study suggests that cytosolic protein kinase system is versatile, androgen-responsive and undergoes developmental changes in normal prostatic tissues; the binding kinetics of R_I may represent a differentiative marker for the autonomous states of the prostatic tumors. (Supported by CA-27418).

0498 CHARACTERIZATION AND SIMULTANEOUS MEASUREMENT OF CYCLIC AMP- AND CYCLIC GMP-DEPENDENT PROTEIN KINASES IN BULLFROG VENTRICULAR MYOCARDIUM, Ronald R. Fiscus, Theodore J. Torphy, Joseph L. Bloom and Steven E. Mayer, Div. Pharmacol., UCSD, LaJolla, CA 92093

Cyclic AMP (cA) and cyclic GMP (cG) have been proposed to mediate the myocardial responses to sympathetic and parasympathetic nerve stimulation, respectively. We have developed an animal model (the in situ perfused bullfrog heart) for studying biochemical responses in ventricular myocardium to autonomic nerve stimulation (The Physiologist 23(4):40, 1980). To study neural regulation of myocardial protein kinases (PK), we have characterized cA-PK and cG-PK in bullfrog ventricles. Using photoaffinity labeling with 8-azido-cA [^{32}P] followed by separation by SDS-polyacrylamide gel eletrophoresis and autoradiography, we have identified two cA-binding proteins (M.W.=46,000 and 55,000 dalton) in soluble and particulate fractions of myocardium. These proteins correspond to the regulatory subunits of Types I and II cA-PK. Most binding occurred in the soluble fraction. Separation of cA-PK isozymes in soluble fractions by DEAE-cellulose chromatography showed that approximately 20% of the kinase activity was in the Type I peak and 80% in the Type II peak. However, the Type II peak also contained cG-PK activity (contributing 15% of total activity). Consistent with a mixed population of cA-PK isozymes, addition of 100 mM NaCl to the homogenizing buffer provided the optimal condition for perserving tissue activation states. In our assay, K_{act} of cA for cA-PK = 30 nM and for cG-PK = 200 nM. K_{act} of cG for cA-PK > 20 μM and for cG-PK = 10 nM. Maximal activation of both kinases occurred with 5 μM cA. Using cA-PK inhibitor (PKI) permitted the simultaneous measurement of both cA-PK (activated by 5 μM cA; inhibited by PKI) and cG-PK (activated by 5 μM cA; not inhibited by PKI). (Supported by HLB 22961 and an MDA Fellowship)

0499 DIFFERENCES IN SENSITIVITY AND DURATION OF EGF-STIMULATED S6 PHOSPHORYLATION, W. Ross Allen, Marit Nilsen-Hamilton and Richard T. Hamilton, Cell Biology Laboratory, The Salk Institute, Box 85800, San Diego, CA 92138

Increased phosphorylation of the ribosomal protein S6 is one of the earliest biological effects of EGF addition to quiescent 3T3 cells. In both Swiss 3T3 and Balb 3T3 cells increases in S6 phosphorylation are observed within minutes but the two cell lines differ in their sensitivity to EGF. Addition of growth factor to these cells increases the rate of turnover of ATP so that the specific activity of ^{32}P-ATP increases 2- to 4-fold. After correction for changes in pool specific activity of the donor phosphate, EGF, and serum stimulate S6 phosphorylation 2-fold in Swiss 3T3 cells and greater than 4-fold in Balb 3T3.

The increase in S6 phosphorylation in Swiss cells given saturating concentrations of EGF or serum is transient, decaying to pre-stimulated levels by 2 - 3 hours. In contrast, Balb 3T3 cells maintain elevated levels of S6 phosphorylation for at least 6 hrs.

The differences in sensitivity and duration of the S6 response to EGF are not due to differences in concentration of surface EGF receptors. Both cell lines bind 70 pg of ^{125}I-EGF per 10^6 cells at 0-4°C, however, they "down-regulate" at different rates. The Swiss 3T3 cells take > 4 hrs to reach a new steady-state level whereas the Balb 3T3 cells take < 2 hrs to reach the same steady-state level of 28 pg ^{125}I-EGF bound/10^6 cells.

The amount of internalized EGF has no effect on S6 phosphorylation. Addition of NH_4Cl or the protonophore nigericin increases the cell-associated ^{125}I-EGF 3-fold over controls by 4 hrs. However, neither of these compounds effects the sensitivity or duration of the EGF stimulated increase in S6 phosphorylation.

0500 CYCLIC AMP-PROTEIN KINASE (cAMP-PK) INDEPENDENT REGULATION OF GLYCOGEN PHOSPHORYLASE (PHOS) IN S49 LYMPHOMA CELLS, Adam H. Kaufman and Laurence L. Brunton, Divisions of Pharmacology and Cardiology, Department of Medicine, UCSD, La Jolla, CA 92093

We have utilized wild type (WT; clone 24.3.2) S49 lymphoma cells and a variant lacking cAMP-PK activity (PK$^-$; clone 24.6.1) to investigate the regulation of PHOS by cAMP dependent and independent processes. Both clones have low content of glycogen (0.8 ± 0.2 μg/mg cells) and PHOS (+AMP: 2.5nmol/min/mg protein). In soluble extracts of the clones, basal PHOS activity ratios (-AMP/+AMP) are ≈0.6. WT cells respond to isoproterenol with a small increase in the activity ratio (from 0.6 to 0.8); PK$^-$ cells are unresponsive. Neither chelation of Ca^{++} (4mM EGTA), stimulation of cAMP degradation (0.3mM imidazole), nor removal of endogenous AMP with charcoal lowers the activity ratio. A decrease of the activity ratio occurs in dilute (1:100) extracts incubated at 30° under dephosphorylating conditions. This inactiviation (-AMP/+AMP: 0.8 → 0.2 in 75 min) is inhibited by β-glycerol-PO_4 (40mM) and NaF (10mM), potentiated by the addition of purified Type I phosphoprotein phosphatase and reversed by the addition of cAMP, PK, and ATP. Contributing to the stability of PHOS *a* in S49 extracts is a soluble, heat stable (95°, 15 min) inhibitor of endogenous phosphatase activity. This inhibitor also blocks PHOS *a* → *b* in rat heart extracts. We conclude that PHOS of S49 cells is regulable by a phosphorylation-dephosphorylation cycle, and that the -AMP/+AMP activity ratio adequately reflects the phosphorylation state. In these cells, PHOS exists predominantly in an active phosphorylated form that occurs independently of cAMP-PK, is insensitive to decreased extracellular Ca^{++}, and may reflect the influence of a potent heat stable inhibitor of phosphoprotein phosphatase activity. (NIH-GM25819, UCCRCC, NIH-GM07752 and RCDA HL00935).

0501 IDENTIFICATION AND PARTIAL CHARACTERIZATION OF MOLECULES RELATED TO EPIDERMAL GROWTH FACTOR, L.M. Matrisian, S.R. Planck and B.E. Magun, Dept. of Anatomy, College of Medicine, University of Arizona, Tucson, Arizona 85724

To better understand the physiological role(s) of the epidermal growth factor (EGF)-receptor system, we examined a number of biological tissues for factors belonging to a putative "EGF-class" of factors defined by the ability to compete with mouse EGF for its plasma membrane receptor. Two such factors were identified in mouse submaxillary gland extracts, and are referred to as Factor I and Factor II. Factor I and Factor II were isolated by the following series of chromatographic steps: BioGel P-10 chromatography, DEAE-cellulose chromatography, gel filtration HPLC, and reverse phase HPLC. In three of the four steps, the factors chromatographed differently from purified mouse EGF. Factor I and Factor II compete with EGF for the binding site on rat embryo fibroblasts and are internalized in a manner similar to that of mouse EGF. Both factors stimulate DNA synthesis in quiescent rat embryo fibroblasts and are capable of inducing anchorage independent growth of the same cell type. The apparent molecular weight of both Factor I and Factor II is approximately 20,000, as determined by gel filtration HPLC. Peptide maps of chymotryptic digests of radiolabeled EGF, Factor I, and Factor II revealed a number of common sequences. However, Factors I and II contained additional spots not seen in the EGF map, and distinct differences between the maps of Factor I and Factor II were noted. Further work is currently underway to determine if the three proteins have a precursor-product relationship, are coded by different alleles of the same gene, or are products of different genes with the same active site.

0502 INSULIN STIMULATES THE PHOSPHORYLATION OF ITS OWN RECEPTOR; M. Kasuga, Y. Zick, D. Blithe, J. Roth and C.R. Kahn; Joslin Diabetes Center, Harvard Univ., Boston, Mass. 02215, and Diabetes Branch and Lab of Mol. Biol., N.I.H., Bethesda, Maryland 20205

To determine if the insulin receptor might undergo phosphorylation, cultured human lymphocytes and rat hepatoma cells were labeled with [^{32}P] orthophosphate and exposed to insulin for different periods of time. The ^{32}P-labeled cells were solubilized in Triton X-100 and the insulin receptor isolated by chromatography on wheat germ agglutinin agarose and immunoprecipitation by a serum containing antibody against insulin receptor. The immunoprecipitates were analyzed by SDS-gel electrophoresis and autoradiographed. Antibody against the insulin receptor specifically immunoprecipitated two ^{32}P-labeled bands with Mr = 95,000 and 68,000. When the cells were incubated with 10^{-6}M insulin, the ^{32}P-labeling of 95K band was selectively increased by about 250% at 1 minute and 350% at 15 minutes. This protein has been identified as the β-subunit of insulin receptor by its immunoprecipitation by several different anti-receptor antibodies and by the fact that it migrates in the same position as the β-subunit of the receptor in reduced and non-reduced SDS-gels. The 95K phosphoprotein was eluted from gels and the phosphoaminoacids were determined by paper electrophoresis after hydrolysis. In the control cells, the only aminoacid phosphorylated was serine; however, after incubation with insulin, both of phosphoserine and phosphotyrosine were identified. These data suggest that an early step in insulin action is phosphorylation of the β-subunit of the insulin receptor and that this reaction may occur on a tyroside residue.

0503 THE DESENSITIZATION OF TYROSINE AMINOTRANSFERASE INDUCTION IN HEPATOMA CELLS BY CYCLIC AMP DERIVATIVES, Jonathan D. Smith and Alice Y.-C. Liu, Dept. of Pharmacology, Harvard Medical School, Boston, MA 02115

The induction of tyrosine aminotransferase (TAT) by 1mM 8Br-cAMP in quiescent H-4 rat hepatoma cells, grown in monolayer culture, peaked at 6 hours and then dropped off approaching basal levels by 16 to 24 hours. The readdition of fresh 8Br-cAMP, 8 to 24 hours after the initial 8Br-cAMP treatment, failed to reinduce TAT activity. The heterologous inducers dexamethasone and insulin also failed to induce TAT after 16 hours of pretreatment with 1mM 8Br-cAMP. Antiserum prepared against purified rat liver TAT was used to determine the relative rates of synthesis and degradation of TAT in control and 8Br-cAMP pretreated H-4 cells. The results indicate that the desensitization could be accounted for by a specific decrease in TAT synthesis. With the exception of a 65,000 dalton protein, the overall pattern of protein synthesis, as determined by SDS polyacrylamide gel electrophoresis of pulse labeled proteins, was similar in the control and 8Br-cAMP pretreated cells. The desensitization of TAT induction was reversible; sensitivity towards dexamethasone was apparent 12 hours after the removal of the cyclic nucleotide. Only active cAMP derivatives (8Br-cAMP and dibutyryl cAMP) were effective in producing the desensitization of TAT induction. The induction of ornithine decarboxylase, an enzyme similar to TAT in its inducibility by both cAMP derivatives and glucocorticoids, was also desensitized by pretreatment of the cells with 8Br-cAMP. This research was supported by NIH grant AM 20274 and NIH training grant GM 07258.

0504 SERUM AND EGF STIMULATED S6 PHOSPHORYLATION AND AITERATION OF mRNA EXPRESSION IN QUIESCENT 3T3 CELLS. George Thomas, Michel Siegmann and Gary Thomas. Friedrich Miescher Institute, Basel, Switzerland

Stimulation of DNA synthesis (measured at 22 hr.) in quiescent 3T3 cells by increasing concentrations of serum or EGF leads to a corresponding increase in the extent of 40S ribosomal protein S6 phosphorylation and protein synthesis (measured at 2 hr.). All three processes reach saturation between 7.5 to 10% serum or between 10^{-10} to 10^{-9}M EGF. However the response elicited by EGF is only a portion of that which is stimulated by serum. In addition to the changes in S6 phosphorylation and protein synthesis, there are also many marked changes in the pattern of translation during this time. Half of these changes are under translational control and are due to the movement of a large pool of stored nonpolysomal mRNA into actively translating polysomes. These changes are also coupled in a dose responsive manner to increased S6 phosphorylation and protein synthesis. Furthermore, following stimulation by serum, polysomes are more highly phosphorylated than either 80S ribosomes or 40S subunits (a selection which we have shown is not due to higher rates of phosphorylation of S6 when present in polysomes), suggesting that the phosphorylation of S6 is involved in facilitating the movement of 80S ribosomes and stored nonpolysomal mRNA into actively translating polysomes. Future experiments in vivo and in vitro are designed to test this model.

0505 PLATELET-DERIVED GROWTH FACTOR STIMULATES TYROSINE-SPECIFIC PHOSPHORYLATION OF A 185 000 DALTON COMPONENT IN HUMAN FIBROBLAST MEMBRANES,

B. Ek, B. Westermark, A. Wasteson and C.-H. Heldin, University of Uppsala, Sweden.

Recent studies have shown that tyrosine-specific protein kinases may be implicated both in virus-induced transformation of cells (e.g. with Rous sarcoma virus) and in cellular growth stimulation by epidermal growth factor (EGF). Platelet-derived growth factor (PDGF), a 30 000 dalton protein, is the major growth promoting factor in serum for connective tissue-derived cells and glial cells. A high-affinity receptor for PDGF has been demonstrated on these cell types. We have investigated if binding of PDGF to its receptor stimulates kinase activity. Incubation of cell membranes from human foreskin fibroblasts with PDGF and ^{32}P-ATP at 0°C led to a rapid incorporation of ^{32}P into a limited number of distinct proteins, the major one having an apparent molecular weight of 185 000. The phosphorylation was dependent of PDGF concentration; it was detectable at 125 ng/ml and optimal at 2.5 µg/ml. The reaction required the presence of either Mg^{2+} or Mn^{2+} ions but was independent of Ca^{2+}. PDGF-stimulated phosphorylation of membrane proteins was also demonstrable in other PDGF-receptor positive cells such as glial cells and 3T3-cells, but not in PDGF-receptor negative cells (e.g. epithelial cells). Analysis of ^{32}P-containing amino acids indicated that PDGF stimulates phosphorylation of tyrosine residues. Thus, although PDGF and EGF bind to separate cell surface receptors they may transmit the mitogenic signal to the nucleus via similar or identical intracellular events, i.e. tyrosine-specific phosphorylation of certain growth controlling proteins.

0506 PURIFICATION OF THE SOLUBILIZED VITELLOGENIN RECEPTOR BY ION EXCHANGE AFFINITY CHROMATOGRAPHY, John W. Woods and Thomas F. Roth, University of Maryland Baltimore County(UMBC), Catonsville, MD 21228

Vitellogenin (VIT) is specifically sequestered into the developing oocytes of all oviparious animals. In the laying hen serum VIT is a dimer of two 250k subunits. After internalization VIT is specifically processed to yield phosvitin (PV) and lipovitelline. The receptor recognizes the PV moiety of the VIT.

Ion exchange-affinity chromatography purification of the solubilized VIT receptor takes advantage of the observation that the solubilized receptor activity is not retained on an ion exchange column. For receptor purification solubilized extracts are preincubated with either PV or VIT and then applied to a DE-52 column. In these experiments the soluble receptor activity is retained on the column, probably in the form of a receptor-ligand complex. This retained material can then be eluted with a high salt containing buffer. In experiments in which the solubilized extract was preincubated with PV, the high salt eluted material now contained PV and two additional proteins of MW 116k and 55k. Trace amounts of several other proteins were also observed. These results are in dramatic contrast to experiments in which the solubilized extract are applied directly to the column. In this case, no proteins were observed in the high salt eluate, We infer from these results that DE-52 binds the soluble receptor ligand complex. We are currently using the high salt eluted material to prepare antibody against the receptor.

0507 GROWTH REGULATION OF Y-1 ADRENAL CORTICAL TUMOR AND OVARIAN GRANULOSA CELLS IN COCULTURE, S.A. Murray, C.V. Byus and W.H. Fletcher, University of California. Riverside, California 92521.

Y-1 adrenal cortical tumor cells in solo culture grow aggressively (doubling time approximately 30 hr.); however, when cocultured with preovulatory phase ovarian granulosa cells, Y-1 cell growth is dramatically reduced, if not eliminated. Coculture conditioned medium does not cause a similar growth reduction, thus, the growth inhibition appears to result from a direct contact between the granulosa and Y-1 cells. Cyclic-AMP can depress the growth rate of Y-1 cells and is thought to communicate between cells via gap junctions. We hypothesized that a cAMP-dependent protein kinase (cAMP-PK) might be involved in the regulation of Y-1 cell growth. A fluorescinated conjugate of the inhibitor protein of free catalytic units, (C) from cAMP-PK was used to follow cytochemically, the subcellular kinetics of kinase dissociation in granulosa and Y-1 cells cultured alone or together. FSH fails to cause kinase dissociation in solo cultured Y-1 cells. There is, however, a time dependent dissociation of C in Y-1 cells contacting FSH stimulated granulosa cells in the coculture. These findings suggest a direct intercellular transfer of an agent capable of causing the dissociation of cAMP-PK could explain the ability of highly differentiated granulosa cells to inhibit the rapid growth of Y-1 tumor cells. (Support NIH HD 13-704; CRCC 79R11 and AM 073 10-02.)

0508 COMPARISON OF REGION-SPECIFIC PROTEIN PHOSPHORYLATION SYSTEMS IN BRAIN, Ivar Walaas, Yale University, New Haven, CT 06510

The regional, cellular and subcellular localizations of most of the brain cyclic AMP- and calcium-stimulated protein phosphorylation systems are incompletely known. We have therefore compared the distribution of the cyclic AMP-, the calcium/calmodulin- and the calcium/phosphatidylserine-stimulated protein kinase activities and of their respective substrates in particulate and soluble fractions from 20 microdissected rat brain regions. All particulate preparations displayed a major calcium/calmodulin-stimulated system, with striking regional differences in both kinase activities and protein substrates. The cyclic AMP-stimulated system displayed cerebellum-specific and basal ganglia-specific particulate substrates, while the calcium/phosphatidylserine-stimulated system was almost undetectable in the particulate preparations. The latter system was highly concentrated in the soluble preparations, but it did not display major regional differences. Similar observations were made on the soluble calcium/calmodulin system, while a considerable number of previously undefined region-specific soluble cyclic AMP-stimulated phosphoproteins were found, particularly in the dopamine-innervated parts of the basal ganglia. Lesion studies suggest that some of the latter proteins may be restricted to specific nerve cell populations, thus indicating involvement in particular functions.

0509 MEASUREMENT OF INTRACELLULAR RECEPTORS FOR CYCLIC GMP, R.L. Hurwitz, M.C. Mumby and J.A. Beavo, University of Washington, SJ-30, Seattle, WA 98195

At least four different proteins have been shown to specifically bind cyclic GMP with high affinity and therefore be implicated as possible intracellular receptors for cyclic GMP. These include 1) cyclic GMP dependent protein kinase, 2) cyclic GMP stimulated cyclic nucleotide phosphodiesterase, 3) light activated cyclic GMP phosphodiesterase, and 4) a protein copurifying with another cyclic GMP phosphodiesterase activity present in platelet and lung tissues. We have produced antisera to bovine preparations of the first two of these proteins and monoclonal antibodies to the first three of them. Crossreactivity studies indicate that all four proteins contain distinct antigenic determinants. The monoclonal antibodies are predominately of the IgG_1 subclass and have affinities of $10^{-9}M$ or higher. Most of the monoclonal antibodies do not inhibit enzyme activity or cyclic GMP binding and therefore can be used directly for immunotitration analysis. Preliminary experiments indicate that less than 10 fmol of phosphodiesterase can be easily measured by an immunoassay procedure utilizing phosphate release from the cyclic nucleotide. Procedures utilizing ^3H cGMP binding are about 2 orders of magnitude less sensitive. These immunoassay procedures allow direct measurement of each cyclic GMP receptor protein to be made in crude preparations containing more than one of the enzymes. They should also be useful in other studies requiring comparison, quantitation and localization of these proteins.

0510 EVIDENCE FOR ACTIVATION OF CHOLESTEROL ESTERASE FROM CORPUS LUTEUM BY A cAMP-DEPENDENT PHOSPHORYLATION PROCESS, K.M.J. Menon and P. Fitzpatrick, Univ. of Michigan, Ann Arbor

Incubation of luteal cells with LH/hCG increases the intracellular accumulation of cAMP, which in turn activates cAMP-dependent protein kinase. To determine an intracellular substrate for cAMP-dependent protein kinase in the ovary, we have examined the phosphorylation of cholesterol esterase, an enzyme involved in the conversion of cholesterol ester to free cholesterol. The preparation used was a 100,000 xg supernatant eluted in the void volume of a Sephadex G-25 column. Preincubation of this fraction for 1 h at 37°C with ATP (1 mM), $MgCl_2$ (1 mM), and 8 Br-cAMP (2.5 μM) resulted in a 70% increase in cholesterol esterase activity over controls. Half-maximal concentrations for stimulation for ATP, cAMP, and 8 Br-cAMP were 0.2 mM, 0.64 μM, and 0.26 μM, respectively. AMP-PNP was unable to substitute for ATP. Pretreatment with E. coli or calf intestine alkaline phosphatase resulted in a 50% decrease in esterase activity. Preincubation with Mg^{2+} resulted in a time-dependent inactivation of the enzyme; this could be partially blocked by the phosphoprotein phosphatase inhibitors, NaF and sodium pyrophosphate. Addition of protein kinase inhibitor to the preincubation mixture decreased the activation by ATP and 8 Br-cAMP 50-90%. Purification of cholesterol esterase approximately 10-fold by pH 5.2 precipitation and elution in the void volume of a Sephadex G-100 column resulted in a preparation that was dependent on added protein kinase for activation. The activation of cholesterol esterase was due to an increase in V_{max}, with no change in the apparent K_m. From this we concluded that ovarian cholesterol esterase is controlled by a cAMP-dependent phosphorylation process.

0511 ALTERED CYCLIC AMP-BINDING PROTEINS IN HUMAN BREAST CANCER, Urs Eppenberger, Klaus Handloser, Willy Küng and Jürg Handschin, Department of Research and Gynecology, University Medical School, 4031 Basel, Switzerland.

Recently the phosphorylation of proteins was discussed as a sensitive index of changes in the cellular environment, and the effects of the protein kinase enzymes are believed to play a role in tumor development. - Investigations have indicated that a relationship exists between intracellular levels of cAMP and cAMP-dependent protein kinases type I and type II during normal and neoplastic growth. This study demonstrates a significant alteration in cAMP-binding protein (cAMP-BP) pattern (R-I and R-II) of human neoplastic mammary tissue (CA) as compared to normal and dysplastic mammary tissues. Fotoaffinity labeling of the cAMP-BP with 8-N_3-/^{32}P/ cyclic AMP revealed on SDS gels that R-I is the major cAMP-BP (49 K). In contrast to CA no proteolytic products (37 K and 36 K) can be detected in normal and dysplastic breast tissue. Only the 52 K cAMP-BP (R-II) is autophosphorylated. All cAMP-BP, except R-II, have apparent $K_D < 10^{-8}$ M and the 37 K and 39 K are not low affinity cAMP-BPs. R-II exhibits an apparent K_D of 10^{-7} M. No difference of K_D could be detected between dysplastic tissue and CA. The relative ratio of R-I versus R-II is significantly increased in normal and dysplastic tissue as compared to CA. It is suggested that in normal tissue cAMP interacts with chromatin through R-I and R-II leading to a specific stimulation of R-I/R-II gene transcription. In CA such selective stimulation might be altered.

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0512 RESISTANCE TO PHOSPHATASE OF THIOPHOSPHORYLATED EPIDERMAL GROWTH FACTOR RECEPTOR IN A431 MEMBRANES, Dan Cassel and Luis Glaser, Washington University School of Medicine, St. Louis, MO 63110

Previous work has established that epidermal growth factor (EGF) increases the phosphorylation of its receptor and other membrane proteins and that these proteins can be rapidly dephosphorylated by membrane-bound protein phosphatase (G. Carpenter, L. King, Jr., and S. Cohen, J. Biol. Chem. 254, 4884, 1979). We report that [^{35}S]adenosine 5'-O-(3-thiotriphosphate) ([^{35}S]ATP$_\gamma$S) is equally effective as [γ^{32}P]ATP as a substrate for the EGF receptor-associated protein kinase in A431 membranes. Both the kinetics and extent of the EGF dependent thiophosphorylation at 0° are similar to those obtained with [γ^{32}P]ATP, provided that ATP hydrolysis by the membrane preparation is inhibited by the addition of adenosine 5'-(β,γ-imino) triphosphate (App(NH)p). At 32° thiophosphorylation is only five times faster than at 0°. The thiophosphorylation reaction requires Mn^{++}, but differs from the phosphorylation reaction in the inability of Mg^{++} to serve as a cofactor. Both EGF-dependent phosphorylated and thiophosphorylated membrane proteins yield the same two major bands of M_r 145-160 x 10^3 in autoradiograms of SDS-polyacrylamide gel electrophorograms. The rate of dephosphorylation of membrane proteins that have been thiophosphorylated in the presence of EGF is dramatically slower (20-40 times) than that of the phosphorylated proteins both at 0° and 32°. This increased metabolic stability of the thiophosphorylated proteins will be useful for investigations of the role of phosphorylation in the biological effects of EGF.

0513 MODULATION OF EPIDERMAL GROWTH FACTOR-DEPENDENT PROTEIN PHOSPHORYLATION IN CELL MEMBRANE PREPARATIONS BY RECEPTOR DOWN REGULATION, J.A. Fernandez-Pol, VA Medical Center and St. Louis University, St. Louis, MO 63125

We have reported previously (Biochemistry 20:3907, 1981; J. Biol. Chem. 256:9742, 1981) that: (a) in normal rat kidney (NRK) membranes there are numerous components whose phosphorylation can be stimulated by epidermal growth factor (EGF). Among these phosphoproteins, two components of Mr 170K and 150K were primarily affected by EGF; and (b) down regulation of EGF receptors in NRK cells by EGF results in a specific decrease in phosphorylation of the 170K- and 150K-dalton membrane components to subsequent stimulation with EGF in vitro. We have recently found that transferrin added to cultured NRK cells potentiates the EGF-dependent in vitro phosphorylation of the 150-170K Mr membrane proteins. This enhancement of the EGF-dependent phosphorylation of the 150-170K Mr membrane proteins by transferrin, was dependent on temperature, transferrin concentration, time, and cell density. These data suggest that modulation of phosphorylation of specific membrane proteins by transferrin may be important in regulating the cells subsequent sensitivity to EGF. In addition, we present the following observations with A431 cells: (a) EGF-induced down regulation of EGF receptors in A431 cells also results in a specific decrease in ^{32}P-phosphorylation of the 170K- and 150K-dalton components to subsequent stimulation with EGF in vitro; (b) immunological studies showed that the reduction of EGF-dependent ^{32}P-phosphorylation of the 150-170K dalton duplet is due to a decrease in the number of available 150-170K dalton molecules in the cell surface membranes. These and other data suggest that the 150-170K dalton phosphoproteins disappear from the cell surface by cellular internalization.

0514 A GROWTH FACTOR ACTIVABLE AND AMILORIDE SENSITIVE Na^+/H^+ EXCHANGER IN FIBROBLASTS - COUPLING TO PROTEIN PHOSPHORYLATION, Jacques Pouysségur, Jean-Claude Chambard and Sonia Paris, Centre de Biochimie, CNRS, Parc Valrose, 06034 Nice, France.

Serum deprivation arrests Chinese hamster lung fibroblast cells (CCl39) in the GO/G1 phase of the cell cycle. Reinitiation of DNA synthesis by serum or the combination of insulin (1-10 µg/ml) with either thrombin, PDGF, FGF is preceeded by very early stimulation of ionic fluxes (Na^+/Rb^+) and protein phosphorylation (27K dalton, 62K dalton and the ribosomal S6 proteins). The synergistic effect of thrombin and insulin seen on DNA synthesis is also observed with the early biochemical events : stimulation of Na^+ influx and phosphorylation of S6. This synergy suggests that a very early convergent pathway exists for these growth factors as well as a possible link between ionic fluxes and protein phosphorylation. We now report that CCl39 cells possess a Na^+/H^+ exchanger in the plasma membrane. This exchanger is specific for Na^+, Li^+ and is highly sensitive to amiloride (half inhibition of H^+ extrusion rate, ID50, is obtained with 3-10 µM amiloride). Interestingly, we found that amiloride also abolished both the growth factor-stimulated Na^+ influx and S6 protein phosphorylation in the same range of concentration.

Therefore, we propose that one of the first post-receptor steps of growth factor action is the activation of an amiloride sensitive Na^+/H^+ exchanger. However, since the dissipation of H^+ gradients, with DNP or CCCP, inhibited growth factor-stimulation of S6 phosphorylation, we suggest that an increase in intracellular pH is one of the key signals delivered by growth factors.

0515 THE EFFECT OF INSULIN ON PROTEIN PHOSPHORYLATIONS IN SARCOLEMMA MEMBRANES FROM SKELETAL MUSCLE, Otto Walaas, Robert S. Horn and Arne N. Wick, Institute of Medical Biochemistry, University of Oslo, Norway, and California Metabolic Research Foundation, La Jolla.

The possibility that membrane protein phosphorylations are initial events in the receptor mediated action of insulin have been studied. Sarcolemma membranes were incubated with 32-P-γ-ATP + Mg in the absence and the presence of insulin and 32-P-labeled proteins identified by PAGE in the presence of SDS. It has been shown that insulin promotes increased phosphorylation of a membrane proteolipid of M_r 15000. After delipidation of the proteolipid a 32-P-labeled subunit of M_r 3500 has been isolated and characterized with respect to amino acid composition. This effect by insulin was attributed to stimulation of a membrane-bound cyclic AMP-independent protein kinase. The stimulatory effect of insulin on this protein kinase activity was enhanced by GTP. Insulin also exerts an effect on the phosphorylation of a membrane protein of M_r 90 000. By incubation of the membrane in the presence of insulin phosphorylation of this protein was decreased. The effect was not dependent upon cyclic AMP. The decreased phosphorylation of this protein due to insulin could be attributed to an increased rate of dephosphorylation.

0516 ROLE OF CYCLIC AMP IN THE INDUCTION OF GLUCAGON RESPONSIVENESS IN CANINE KIDNEY CELLS. Suzanne K. Beckner and Michael C. Lin, NIH, Bethesda, MD 20205.

MDCK cells transformed with Harvey sarcoma virus selectively lose glucagon receptors and responsiveness as compared to the parental line. Glucagon responsiveness can be restored to these cells with butyrate, a process which requires the de novo synthesis of protein. Glucagon responsiveness can also be induced in transformed cells with prostaglandin E_1 (PGE_1), a potent activator of MDCK cell adenylate cyclase, and RO 20-1724, an inhibitor of phosphodiesterase (PDE), but not by cholera toxin. In addition, exogenous cyclic AMP (1 mM) prevents the PGE_1 and RO 20-1724 mediated induction. The cyclic AMP response of transformed cells to PGE_1 is maximal at 10 nM and half maximal at 1 nM, while its effect on induction is undetectable at 1 nM, maximal at 10 nM and declines at higher concentrations. Similarly, induction of glucagon responsiveness by RO 20-1724 is also biphasic, being maximal at 100 nM and declining at both higher and lower concentrations, while its K_i for PDE is 100 nM, with no detectable inhibition below 1 nM, where partial induction of glucagon responsiveness is still evident. The data suggest that while cyclic AMP levels are increased during maximal induction by PGE_1 and RO 20-1724, cyclic AMP does not totally mediate the induction of glucagon responsiveness in transformed cells and higher concentrations of cyclic AMP may actually inhibit the induction by these agents.

0517 DIBUTYRYL CYCLIC AMP INDUCED EXPRESSION OF A CHEMOTACTIC RECEPTOR DURING MYELOID DEVELOPMENT, James Niedel and Thomas Chaplinski, Duke University Medical Center, Durham, N.C. 27710

Human promyelocytic leukemia cells (HL -60) differentiate in tissue culture after treatment with analogs of cyclic AMP or substances which raise intracellular levels of cAMP. The earliest observable change is the synthesis and expression on the plasma membrane of the formyl peptide chemotactic receptor. The increase in surface receptor can be detected within 2 hrs. after treatment and continues linearly for 48-72 hrs., reaching a maximum density of 250,000 receptors/cell; a density 5-fold greater than that seen on peripheral blood neutrophils. Cycloheximide (0.3-3 μg/ml) causes a parallel inhibition of protein synthesis and receptor expression. The constant presence of cAMP is necessary for receptor expression and no finite commitment event occurs. The superoxide response and cell adherence develop on day 2 and are maximal by day 4. Morphological changes also occur after day 2, but few of the cells develop beyond the metamyelocyte stage. At 24 hrs., the receptor is coupled to the mechanism for lysosomal enzyme secretion. Receptors for insulin, phorbol dibutyrate, β-adrenergic ligands and the Fc portion of IgG do not change during cAMP induced maturation.

0518 HORMONE RECEPTOR EVOLUTION IN THE OVARIAN GRANULOSA CELL: INDUCTION BY CYCLIC AMP Michael Knecht and Kevin Catt, Endocrinology and Reproduction Research Branch, NICHD, Bethesda, MD. 20205

The granulosa cell of the ovarian follicle provides a valuable model for studies on hormone-induced cellular differentiation. During follicle maturation, FSH induces and maintains receptors for LH, prolactin, and gonadotropin-releasing hormone (GnRH), and these actions of FSH are expressed in vitro during 2-day culture of undifferentiated granulosa cells from ovaries of hypophysectomized estrogen-treated immature rats. This induction by FSH of plasma membrane receptors was reproduced by 8-Bromo-cAMP and agents which activate adenylate cyclase, such as cholera toxin and prostaglandins. The concentration-response curves for both FSH and cholera toxin indicated that amounts of the agonist which elevated LH receptors also stimulated adenylate cyclase activity. Submaximal concentrations of hormones added together were supra-additive for cAMP and LH receptor production, while hormone levels stimulating maximal bioactivity alone were not additive in combination. Conversely, peptide inhibitors of FSH-induced cAMP accumulation, such as GnRH or EGF, also prevented LH receptor formation in a concentration-dependent manner. The inhibition of both cAMP and LH receptor formation by GnRH and EGF occurred during the second day of culture, and was caused by increased cAMP catabolism and decreased cAMP biosynthesis, since these peptides enhanced phosphodieterase activity and reduced adenylate cyclase activity in FSH-treated cell cultures. These results indicate that gonadotropic induction of heterologous hormone receptors in the ovarian granulosa cell occurs through cAMP-dependent pathways, and that inhibitory peptides such as GnRH and EGF prevent receptor formation by impairing cAMP accumulation.

0519 COMPARISON OF NEUROSPORA AND MAMMALIAN CYCLIC AMP CONTROL SYSTEMS
Martin L. Pall, Gary Rosenberg, and James M. Trevillyan
Washington State University, Pullman, WA 99164

Three properties of the cyclic AMP control system of the fungus Neurospora crassa show striking similarities to the mammalian control system. The membrane-bound adenylate cyclase of Neurospora shows a magnesium-dependent, guanine nucleotide-stimulated activity which is more labile than is the manganese dependent activity. These results show that the Neurospora enzyme has a guanine nucleotide-binding component which is similar to but more labile than the mammalian G/F component. The cyclic AMP-dependent protein kinase of Neurospora also closely resembles the mammalian enzyme. The regulatory subunit of Neurospora is very similar to the mammalian type I regulatory subunit in subunit molecular weight (47,000), pI (5.4), number of cyclic AMP binding sites (2), Stokes radius (46 A°), and other physical properties. Finally, evidence will be presented suggesting that cyclic AMP stimulation of glycogenolysis occurs in Neurospora. All three of these studies suggest that the cyclic AMP control system has been highly conserved in eukaryotic evolution.

0520 cAMP IN THE DEVELOPMENT OF THE SIMPLE EUKARYOTE DICTYOSTELIUM DISCOIDEUM, Ben H. Leichtling, Irene Majerfeld, Donna S. Coffman and Howard V. Rickenberg, National Jewish Hospital & University of Colorado Health Sciences Center, Denver, CO 80206

The cellular slime mold Dictyostelium discoideum grows as unicellular amoebae. Deprivation of nutrients initiates a developmental program which includes the formation of multicellular aggregates and the differentiation of the ameobae into two cell types, spores and stalk cells. cAMP acts both as the chemotactic agent and as intracellular effector of development. We have identified a membranal protein of 42,000 daltons which can be photolabeled with P^{32}-8-N_3GTP and also is a substrate for cholera toxin-stimulated ADP-ribosylation. This putative G-protein, unlike cell surface receptors and adenylate cyclase activity which increase greatly during development, is present at the same level in growing and developing amoebae. The G-protein may play a role, additional to that of communicating cAMP receptor occupancy to the adenylate cyclase, in vegetative amoebae.
A cAMP-dependent protein kinase has been partially purified from developing cells and changes in the kinase subunits during development. The molecular weights of the regulatory and the catalytic subunits, both in crude extracts and in the partially purified preparations, are different from their mammalian counterparts. A heat-stable proteinaceous inhibitor of the kinase has been isolated from the ameobae.
The role of the regulation of the adenylate cyclase and of the cAMP-dependent protein kinase in the development of Dictyostelium as well as possible evolutionary implications will be discussed.

0521 STOICHIOMETRY AND PHOSPHOPEPTIDE ANALYSIS OF EGF-INDUCED EGF RECEPTOR PHOSPHORYLATION IN PURIFIED A431 MEMBRANES, Hilda Chamras, Robin Yeaton, Shintaro Iwashita and C. Fred Fox, Department of Microbiology and The Molecular Biology Institute, University of California, Los Angeles, CA 90024

Incubation of purified A431 cell surface membranes with γ-^{32}Pi-ATP and EGF leads to phosphorylation of protein(s) having the same molecular weight as peptides that are affinity labeled with EGF. This phosphorylated fraction fraction has been referred to as "EGF receptor" in a number of studies. In membrane preparations characterized by a doublet receptor band, 145/160 kdaltons, the stoichiometry of receptor phosphorylation is relatively low (< 0.5), but is increased to greater than 4 in preparations in which receptor migrates as a single band at the higher (160 kdalton) molecular weight. Two dimensional fingerprinting analysis of tryptic P-peptides produced from receptor phosphorylated in membranes reveals homology in the 145 and 160 kdalton peptides, but certain of the 11 major P-peptide fragments present in the 160 kdalton peptide are present in reduced amount or absent in the 145 kdalton peptide. Other information, including influences of detergent on phosphorylation stoichiometry and receptor P-peptide maps will be presented. (Supported by grants from USPHS, ACS and MDAA).