GENETIC AND IN VITRO ANALYSIS OF CELL COMPARTMENTALIZATION

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Genetic and in Vitro Analysis of Cell Compartmentalization

Keynote Address

H 001 PROTEIN IMPORT INTO MITOCHONDRIA

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During recent years considerable insight has been gained into the transport pathways of nuclear-encoded proteins into mitochondria. Since mitochondria are divided into a number of subcompartments these pathways are rather diverse and of different complexity.

It would appear that the most complex import pathway is used by those proteins which function in the mitochondrial intermembrane space. Transport into other compartments may be considered as shorter versions thereof. Precursors of intermembrane-space (IMS) proteins such as cytochrome c_1, or cytochrome b_2 are first recognized by surface proteins of the outer membrane (OM). Then insertion occurs into the OM with amino terminal parts of the precursor. As a next step the amino-terminal matrix targeting sequence becomes translocated across the inner mitochondrial membrane (IM) with the help of the mitochondrial membrane potential ($\Delta\Psi$) and subsequent transfer of the rest of the polypeptide chain takes place through translocation contact sites (TCS). At these TCS OM and IM are closely apposed so that a segment of about 40 amino acids is sufficient to span both membranes. Transfer through TCS usually requires ATP in the cytosolic compartment for unfolding of the precursor. At the inner side of the IM, the newly imported polypeptide chain interacts with the mitochondrial stress protein, hsp60. Folding of the polypeptide, which depends on ATP-hydrolysis, may occur at this step, however, no release. The matrix targeting sequence is cleaved off by the mitochondrial processing peptidase (MPP). Then the protein becomes retranslocated across the inner membrane in a reaction which resembles that of prokaryotic export of polypeptides. A re-export signal sequence at the amino terminus is believed to direct the protein into the IMS where cleavage of this second signal sequence occurs and where heme addition and final assembly take place.

Precursors whose final location is the matrix space, use only the first transfer step. They have only matrix targeting signals and after movement through TCS, cleavage by MPP, folding and assembly at hsp60, they remain in the matrix space. Proteins of the IM may be released either at the level of TCS into the IM (e.g. the ADP/ATP carrier), or may follow a pathway similar to that of IMS proteins, namely through the matrix space (e.g. subunit 9 of F_0-ATPase).

With a few proteins exceptional pathways have been observed, the most prominent example being cytochrome c. This component of the IMS is not made with cleavable signal sequences and becomes directly translocated across the OM into the IMS whereby the process of covalent heme additon is playing a decisive role.

More recently, it has been possible to identify and characterize a number of the components of the complex protein translocation machinery. We will discuss two components of the OM, MOM19 and MOM72, which act as surface receptors; a component of the OM, MOM38, which has many characteristics of a general insertion protein; MPP and PEP, two proteins of the matrix which cooperate in the cleavage of matrix targeting signals; and hsp60 which appears to have an essential role in facilitating the correct folding of newly imported polypeptide chains and the oligomerization of enzymes in the matrix and IM.

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Mechanisms of Protein Targeting and Translocation

H 002 INSERTION AND TOPOLOGY OF MEMBRANE PROTEINS, Bernhard Dobberstein, Joachim Lipp, Stephen High and Oddmund Bakke, EMBL, Postfach 10.2209, D-6900 Heidelberg, Federal Republic of Germany.

Signal sequences mediate translocation of protein across, or insertion into, the membrane of the endoplasmic reticulum (ER). Three types of topogenic signals can be distinguished in secretory or membrane spanning proteins: cleaved signal sequences, uncleaved signal sequences (signal-anchor sequences) and stop-transfer sequences. Structural properties of these signals and how they determine the orientation of proteins in the ER membrane have been investigated with mutant proteins in a cell free system. We conclude from our studies that a functional signal sequence has a tripartite structure: a hydrophobic core region that mediates targeting and insertion into the ER membrane, and flanking polar segments that determine cleavage by signal peptidase and orientation of a protein in the membrane. Proteins that span the membrane several times can be assembled from identical signal-anchor sequences, suggesting that the relative position of a topogenic signal in a polypeptide determines signal or stop-transfer function.

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H 003 TRANSPORT OF PROTEINS INTO PEROXISOMES, Suresh Subramani, Department of Biology, B-022 Bonner Hall, University of California San Diego, La Jolla, CA 92093.

The enzyme luciferase from the firefly Photinus pyralis is targeted to peroxisomes in mammalian, plant, firefly and yeast cells. A C-terminal tripeptide (Ser-Lys-Leu), initially identified in luciferase, is both necessary and sufficient for peroxisomal targeting of proteins in diverse species. Mutational analysis has revealed a consensus peroxisomal targeting signal (PTS) that is conserved at the C-terminus of at least 16 peroxisomal proteins from diverse species. Some peroxisomal proteins contain the consensus tripeptide at internal locations only, but the ability of this sequence to function at internal locations is still being tested. Independent immunological evidence for the conservation of the PTS has come from the generation of anti-PTS antibodies which recognize peroxisomes specifically in diverse species.

H 004 SIGNAL RECOGNITION AND PROTEIN TARGETING, Peter Walter, Harris Bernstein, Byron Hann, Stephen Ogg, Mark Poritz, Dieter Zopf, Department of Biochemistry and Biophysics, University of California, Medical School, San Francisco, CA 94143-0448.

Protein targeting to the endoplasmic reticulum (ER) membrane in mammalian cells is catalyzed by the signal recognition particle (SRP). Signal sequences emerging as part of nascent secretory proteins from the ribosome bind to the 54kDa SRP subunit (SRP54). Sequence analysis of SRP54 revealed a strong homology to the α-subunit of the SRP receptor (SRα) (1, 2), a heterodimeric membrane protein of the ER also involved in the targeting reaction. Recently, it was shown by Reid Gilmore and co-workers that the SRP receptor is a GTP binding protein (3). The homology between SRP54 and SRα is restricted to the domain which contains the GTP binding pocket, but is not limited to only the residues directly involved in GTP binding. A second domain on SRP54 contains 11% methionine residues which might contribute, due to their flexible side chains, to a binding site with sufficient plasticity to accommodate signal sequences dispite their lack of primary sequence homology.

Although there is presently no direct functional evidence for an SRP-like activity involved in protein targeting in yeast or bacteria, proteins which are homologous to SRP54 and SRα have been identified by data bank searches in E. coli (1, 2) and by a PCR approach in S. cerevisiae and S. pombe. While the function of these gene products is still unknown, gene disruption experiments indicate that both genes are essential for growth in S. cerevisiae (4, unpublished). Our progress regarding the elucidation of the structure and function of these proteins will be discussed.

Phylogenetic studies show that E. coli 4.5S RNA shares a homologous domain with mammalian SRP RNA (5, 6). We have constructed a mutant 4.5S RNA which is dominant lethal. After induction of the dominant lethal 4.5S RNA the earliest observed phenotype is a full and permanent induction of the heat shock response, followed by a pathological separation of outer and inner membrane. These phenotypes indicate possible defects in membrane assembly. The secretion of periplasmic proteins does not seem to be affected.

Taken together our findings imply that essential components (SRP RNA, SRP54, SRα) and features of the targeting mechanism may be conserved from bacteria to mammals.

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H 005 ENZYME MECHANISMS OF PROTEIN TRANSLOCATION ACROSS BIOLOGICAL MEMBRANES, Marilyn Rice, Douglas Geissert, and William Wickner, Molecular Biology Institute and Department of Biological Chemistry, University of California, Los Angeles, CA 90024-1570. We are purifying each of the essential components for protein transfer across the plasma membrane of E. coli. Thus far, this includes precursor proteins, chaperone proteins which prevent their aggregation in the cytoplasm, the SecA protein [a peripheral membrane ATPase which couples the energy of ATP hydrolysis to protein export], and leader peptidase [which cleaves the leader peptide once transit is complete]. We have developed assays for the relevant functional integral membrane proteins, including SecY protein. Current concepts of the interplay of these proteins to facilitate protein secretion and membrane assembly will be discussed.

Molecular Genetics of Translocation and Secretion

H 006 PROTEIN FOLDING IN THE ENDOPLASMIC RETICULUM, Mary-Jane Gething, Kenji Kohno, Yasunori Kozutsumi, Karl Normington, Mark Segal and Joseph Sambrook, Department of Biochemistry and Howard Hughes Medical Institute, UT Southwestern Medical Center, Dallas, Texas 75235.

The folding and assembly of influenza hemagglutinin (HA) is a rate-limiting step in transport of HA from the ER[1,2]. Unfolded HA molecules are associated with BiP[1], a cellular ER protein that has been shown to correspond to the glucose-regulated protein GRP78[3,4]. Observations that BiP binds transiently to a variety of wild-type membrane and secretory proteins, and more permanently to malfolded proteins that are trapped in the ER, has led to the hypothesis that BiP plays a role in folding in the ER lumen. Cloning and sequencing of cDNAs encoding mammalian[4-6] and yeast[7,8] BiPs has demonstrated that BiP is a member of the HSP70 protein family. The yeast BiP gene is essential for cell viability[7,8], and like its mammalian counterpart can be induced by the accumulation of unfolded proteins in the ER[8,9]. We have now investigated the nature of the recognition of nascent HA polypeptides by BiP and have identified a species that appears to be a transient BiP-HA heterodimer. Analysis of the interaction of BiP with HA mutants that lack various structural domains has demonstrated that BiP binds to amino acid sequences that in the folded HA molecule form the trimeric stalk domain. Our current working hypothesis is that BiP stabilizes folding intermediates by binding to exposed hydrophobic structures. BiP is displaced when these hydrophobic structures become buried upon folding of the protein into its correct tertiary or quaternary conformation, or upon formation of malfolded aggregates.

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H 007 **REGULATION OF VESICULAR TRANSPORT BY A GTP-BINDING PROTEIN**, Peter Novick, Nancy C. Walworth, Alisa K. Kabcenell, Robert Bowser, Heike Muller and Jaygopal Nair, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510

At each stage of the secretory pathway, membrane and lumenal components are transported by the budding and fusion of carrier vesicles. The specificity of the vesicle targetting reaction must be high to maintain the orderly flow of material in the cell. Ten *SEC* gene products are required for the post-Golgi stage of the yeast secretory pathway, but are not required for earlier vesicular transport events. Therefore, these genes may encode proteins which maintain the specificity of transport to the cell surface. One of them, *SEC4*, encodes a GTP-binding protein. Pulse-chase studies (1) have shown that the Sec4 protein goes through a cycle of localization; it is synthesized as a soluble protein, then binds rapidly to the surface of secretory vesicles which subsequently fuse with the plasma membrane. The protein can recycle from the plasma membrane to a new round of vesicles, possibly through a soluble intermediate. Site-directed mutagenesis studies suggest that the cycle of localization may be obligatorily coupled to a cycle of binding and hydrolysis of GTP (2). Biochemical studies on purified Sec4 protein imply that additional proteins are necessary to control the exchange of GTP onto Sec4, the hydrolysis of the bound nucleotide and the attachment of Sec4 protein to vesicles. *SEC4* displays strong genetic interactions with several of the late acting *SEC* genes including *SEC2* and *SEC15* (3). The Sec15 protein is 105 kD and is peripherally associated with the inner surface of the plasma membrane. Overproduction of Sec15 inhibits cell growth and leads to the formation of a patch of Sec15 protein and a cluster of secretory vesicles (4). The ability of Sec15 to form a protein patch upon overproduction is dependent on the function of Sec2 and Sec4, but not on the function of the other late acting *SEC* gene products. Sec15 may normally act in conjunction with Sec2 to dock secretory vesicles bearing Sec4 onto the plasma membrane, thereby maintaining the specificity of the exocytotic fusion reaction.

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H 008 NUCLEAR FUSION IN YEAST, Mark D. Rose, Joseph P. Vogel, Pamela B. Meluh, and Leanne D. Misra, Dept. of Biology, Princeton University, Princeton, NJ, 08544

In *S. cerevisiae* the nuclear envelope remains intact throughout mitosis, meiosis and conjugation. Therefore, formation of diploid nuclei during conjugation requires nuclear fusion (karyogamy). Karyogamy is dependent upon three *KAR* genes, *CDC4*, activation by yeast mating hormone, and intact microtubules.

The *KAR2* gene encodes the yeast homologue of BiP/GRP78, the ER resident member of HSP70 family. We have used a set of recessive temperature sensitive mutations in *KAR2* to analyze the normal in vivo function of BiP. One class of mutations, exemplified by the *kar2-159* allele, causes a rapid loss of viability after shift to the high temperature. With similar rapid kinetics, the precursors of several secreted proteins accumulate in the cytoplasm. The same results are obtained by shutting off synthesis of BiP from the wild type *KAR2* gene. Therefore, *in vivo*, BiP plays an essential role in the translocation of secreted proteins into the ER.

Other mutant alleles of *KAR2* block cell growth at high temperature without concomitant loss of viability. One example of this is the original mutation *kar2-1*. Analysis of this class of mutant reveals that most of these mutations have no effect on translocation. Examination of secreted proteins reveals an additional requirement for *KAR2* in a later stage in the secretory pathway.

The predicted *KAR3* protein (729 amino acids) bears a striking relationship with *Drosophila* kinesin. Like kinesin, *KAR3* has three domains: a small (110 aa) N-terminal globular domain, a central (270 aa) coiled-coil alpha-helical region, and a large (349 aa) C-terminal globular domain. The large domain is homologous to the ATP and microtubule binding domain of kinesin heavy chain (37% identity). Remarkably, the order of the domains is reversed with respect to kinesin. Fusion proteins and a semi-dominant mutant protein, *kar3-1*, localize specifically to the extranuclear microtubules. We speculate that *KAR3* interacts with microtubules and like kinesin, is a microtubule-based motility protein.

KAR3 is expressed at low levels in all cell types, but is induced at least 20-fold by mating pheromone. Although, *KAR3* is not an essential gene, strains bearing a deletion grow slowly, become blocked in G2, demonstrate chromosome instability and fail to sporulate. Thus *KAR3* plays an important ancillary role in mitosis as well as karyogamy.

Genetic and in Vitro Analysis of Cell Compartmentalization

Mechanisms of Protein Sorting to Lysosomes

H 009 VACUOLAR PROTEIN SORTING IN YEAST: POTENTIAL ROLE FOR A PROTEIN KINASE
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The yeast vacuole is a lysosome-like organelle that contains a variety of hydrolases including the protease carboxypeptidase Y (CPY). CPY transits through early compartments of the secretory pathway prior to being sorted and targeted for final delivery to the vacuole. To identify gene functions that participate in the sorting and transport of this and other vacuolar proteins, we have isolated mutants that exhibit defects in vacuolar protein sorting (*vps*)[1,2]. In *vps* mutants, the Golgi-modified precursor form of CPY is secreted from the mutant cells.

Toward a molecular understanding of the roles *VPS* gene products play in this transport event, we have cloned and sequenced five of the *VPS* genes. Results obtained with two of these, *VPS*15 and *VPS*33, have suggested that protein phosphorylation may play a role in the regulation of this process. Mutations in either of these two genes lead to severe defects in the sorting of CPY as well as other vacuolar proteins. Mutant alleles of both *VPS*15 and *VPS*33 genes that result in a temperature-sensitive growth phenotype have been isolated. The *VPS*15 and 33 genes were cloned by complementation of the *ts* growth phenotype. Integrative mapping has confirmed that each clone corresponds to the correct chromosomal *VPS* locus.

Based on the DNA sequence, *VPS*15 contains an open reading frame of 4,227 nucleotides, sufficient to encode a protein of 1409 amino acids, and *VPS*33 is predicted to encode a protein of 691 amino acids. Polyclonal antisera raised against open reading frame sequences from *VPS*15 and 33 detect proteins of the expected size in extracts from wild type yeast cells. Neither protein appears to be glycosylated. Immunofluorescence data indicates that the Vps33p associates with the cytoplasmic surface of the vacuole membrane. The Vps15 protein fractionates with yeast membranes, contains a short amino-terminal consensus sequence for myristic acid modification, and can be labelled in vivo with ^{3}H-myristic acid.

The deduced Vps15p sequence shares significant sequence similarity with the catalytic subunit of Ser/Thr protein kinases. Site-directed mutations in the *VPS*15 gene that result in single amino acid changes in highly conserved kinase domains eliminate complementing activity of the cloned gene. Potential phosphorylation of Vps15p as well as other *VPS* gene products has been analyzed by in vivo labelling with $^{32}PO_4$. These studies have shown that the Vps33p is a phosphoprotein and its phosphorylation appears to be dependent on the presence of a functional *VPS*15 gene. Together, these observations indicate that sorting and/or transport of at least some vacuolar proteins may be regulated by the specific phosphorylation (activation) of components of the sorting machinery. We are attempting to test this hypothesis directly using an in vitro transport assay that reconstitutes a late step in the sorting of precursor CPY to the vacuole.

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H 010 GENERATION OF A GlcNAc-P-TRANSFERASE RECOGNITION MARKER IN THE SECRETORY PROTEIN, PEPSINOGEN,
Thomas J. Baranski, Phyllis L. Faust, and Stuart Kornfeld, Department of Hematology/Oncology, Washington University School of Medicine, St. Louis, MO 63110

We are investigating the nature of the protein determinant of lysosomal enzymes which allows them to interact with the enzyme UDP-GlcNAc: glycoprotein N-Acetylglucosamine-1-P phosphotransferase (GlcNAc-P-transferase). The GlcNAc-P-transferase catalyzes the initial reaction to generate a mannose 6-phosphate on the oligosaccharides of lysosomal enzymes which then bind to a mannose 6-phosphate receptor and are targeted to the lysosome. We are investigating the human aspartyl proteases cathepsin D, a lysosomal enzyme, and pepsinogen, a secretory protein. When the corresponding RNAs are microinjected into Xenopus oocytes, cathepsin D acquires mannose 6-phosphate and is retained intracellularly. A glyco- form of pepsinogen is not phosphorylated and is secreted, demonstrating that pepsinogen lacks the GlcNAc-P-transferase recognition marker. The structural homologies and the amino acid sequence similarities between cathepsin D and pepsinogen have allowed chimeric molecules to be constructed which substitute regions of cathepsin D into glycopepsinogen while preserving the conformation of the donor cathepsin D region. This approach has identified two noncontiguous regions of cathepsin D which generate a functional GlcNAc-P-transferase recognition marker when substituted into glycopepsinogen. Examination of the crystal structure of pepsinogen reveals that these two regions are in direct apposition. Our results demonstrate a unique and interesting form of a "patch recognition marker" that is presumably shared among over fifty different lysosomal enzymes. Moreover, in at least one of the two regions, a single lysine has been specifically substituted instead of cathepsin D sequence to generate a functional recognition marker.

H 011 PROTEIN TARGETING TO THE YEAST VACUOLE, Tom H. Stevens, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

Proteins destined for delivery to the yeast lysosome-like vacuole transit the endoplasmic reticulum and Golgi apparatus. Our work has focused on dissecting the assembly pathway of the yeast vacuole and understanding the mechanism of targeting of vacuolar proteins. Using a mutational approach we have identified a small protein domain on carboxypeptidase Y (CPY) that is essential for the targeting of this protein to the vacuole. Mutational analysis indicates that four contiguous residues, Gln•Arg•Pro•Leu, are critically important for the functioning of the CPY vacuolar targeting signal. To identify the CPY sorting receptor we have isolated mutations that compensate for the CPY missorting mutations (eg. Gln→Lys). At least one of these extragenic suppressors is allele specific (ie. suppresses Gln→Lys, but not Pro→Arg), and is thus a good candidate for a mutation giving rise to a CPY sorting receptor with altered binding specificity.

In our analysis of the Golgi to vacuole transport machinery we have isolated a large collection of yeast mutants (vps) that fail to correctly sort vacuolar proteins. To characterize the role of individual VPS gene products we have developed an in vitro assay for Golgi to vacuole transport. Using a yeast semi-intact cell system the transport of proCPY from the Golgi to the vacuole is monitored by following the conversion of proCPY→mature CPY. This cell-free reaction requires the addition of a cytosol fraction, ATP and an ATP-regenerating system, and exhibits a sharp temperature dependence. We are currently testing cytosolic and membrane fractions from vps mutants for their ability to function in this in vitro assay.

Phenotypic analysis of the vps mutants revealed that two of the mutants were defective for acidification of the vacuole, and contained greatly reduced levels of the vacuolar H^+-ATPase. These results suggest that acidification of the vacuolar network may be required for proper vacuolar protein sorting. To directly test this hypothesis we have cloned the structural gene (VAT2) for the 60 kD H^+-ATPase subunit, and constructed vat2 null mutants. Vat2 yeast cells grow more slowly than wild type, they lack the entire vacuolar H^+-ATPase complex, and their vacuolar lumenal pH is 7.1. The phenotypic consequences of this lack of vacuolar acidification are under analysis.

Protein Retention and Organelle Selectivity

H 012 CHARACTERIZATION OF CLATHRIN HEAVY CHAIN'S ROLE IN THE INTRACELLULAR RETENTION OF GOLGI MEMBRANE PROTEINS, Gregory S. Payne, Judith A. Finlay, Mary A. Seeger and Kathleen Wilsbach, Department of Biological Chemistry, UCLA School of Medicine and Molecular Biology Institute, UCLA, Los Angeles, CA 90024.

Clathrin-coated membranes have been implicated as sites of protein sorting and membrane vesiculation during intracellular protein transport. To investigate the function of clathrin in these processes, we have generated viable, slow-growing mutants of the yeast *Saccharomyces cerevisiae* which lack clathrin heavy chain. Recent results have focused our attention on clathrin's role in orchestrating maturation of the peptide pheromone α-factor as it is secreted from cells. The biosynthetic pathway of α-factor follows the paradigm of peptide hormone maturation and secretion by eukaryotic cells: the hormone is synthesized as part of a larger precursor which is proteolytically processed during transport through the secretory pathway. Proteolytic maturation of α-factor precursor occurs as the polypeptide passes through the Golgi body and is packaged into secretory vesicles. Characterization of α-factor production by *chc1* and *CHC1* strains revealed that mutants export a highly glycosylated form of the precursor that has not been proteolytically processed. The defect in α-factor processing can be accounted for by the finding that the protease which initiates maturation in the Golgi apparatus, the *KEX2* endoprotease (Kex2p), is mislocalized to the cell surface in *chc1* cells. Based on this observation we suggest that, in wild-type cells, clathrin serves to retain Kex2p in the Golgi apparatus. In order to determine whether clathrin plays a role in the localization of other Golgi membrane proteins we are determining whether the intracellular retention another α-factor maturation enzyme, dipeptidyl aminopeptidase A, is also perturbed in *chc1* cells. Analysis of the subcellular distribution of both maturation enzymes is being carried out using cells carrying a temperature-sensitive allele of *chc1*. In this fashion, the immediate effects of a loss of clathrin heavy chain function can be ascertained. Preliminary results suggest that, within an hour after shift to the non-permissive temperature, the mutant cells secrete α-factor precursor and mislocalize Kex2p to the cell surface. Finally, the secretion of α-factor precursor is being used as a genetic screen to isolate yeast mutants defective in clathrin assembly.

H 013 GENES REQUIRED FOR RETRIEVAL OF RESIDENT ER PROTEINS FROM THE GOLGI, Hugh R.B. Pelham, Neta Dean, Kevin G. Hardwick, Michael J. Lewis, Jan Semenza and Deborah J. Sweet, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England.

Resident proteins of the ER lumen are retained in cells by a mechanism that involves a C terminal signal sequence (KDEL in animal cells, HDEL in *Saccharomyces cerevisiae*). The retained proteins are able to leave the ER and, in yeast, are subject to Golgi-specific carbohydrate modifications. The acquisition of these modifications is dependent on the activity of the *SEC18* gene product, which is required for fusion of transport vesicles with their target membrane; thus, sorting of ER residents from secretory proteins occurs in a compartment topologically distinct from the ER. They must then return to the ER by vesicular transport.

Several yeast genes that affect the sorting process have been identified. These include some of the *sec* genes that are required for ER-to-Golgi transport (and probably for other transport steps). The absence of one gene (*erd1*) causes a defect in the Golgi that affects the normal modification of glycoproteins and interferes with the retrieval process. The *ERD1* product is a 43 kD integral membrane protein. Another gene (*erd2*) appears to be required specifically for the transport of HDEL-containing proteins from Golgi to ER; overexpression of this gene causes retrieval to occur from an earlier point on the secretory pathway than normal. *ERD2* encodes a 26 kD hydrophobic, probably integral membrane protein that is essential for growth.

H 014 RETENTION OF A TRANSMEMBRANE ER PROTEIN, Per A. Peterson, Michael Jackson and Tommy Nilsson, Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037. Most adenoviruses evade immune surveillance by expressing a small, glycosylated membrane protein that binds to MHC class I antigens. The adenoviral glycoprotein, derived from the E3 region of the virus, is located in the endoplasmic reticulum (ER) of infected cells, where it retains newly synthesized class I molecules. We have examined the structural features of the viral protein, termed E19, to identify a motif responsible for its subcellular distribution. Truncations of the 15 amino acid long cytoplasmic tail promoted exit from the ER. The cytoplasmic tail sequence is not only necessary but sufficient for retention in the ER, as demonstrated by the fact that several plasma-membrane proteins become retained in the ER if the COOH-terminal sequence of E19 is appended. A series of chimeric constructs were engineered to examine the size of the structural motif, its position dependence and its sequence requirements. The six terminal residues were sufficient to induce retention but they have to occupy the extreme COOH-terminal position to be functional. Similar size COOH-terminal sequences from five endogenous, ER-retained transmembrane proteins could also induce retention of marker proteins if positioned in the COOH-terminal position. An extensive series of point mutations identified the common denominator for retention as two lysines in positions -3 and -4 or -3 and -5.

To address the question whether retention in the ER is accomplished by "retention proper" or by retrieval from a compartment subsequent to the ER in the exocytotic pathway, we have examined the post-translational modification of a marker protein endowed with the E19-structural motif. Since this retained protein becomes o-glycosylated (as does the truncated, SEKDEL-containing counterpart) and acylated with palmitic acid, our working hypothesis is that ER proteins are transported to a compartment other than the ER, where active retrieval of ER proteins occur. Proteins destined for other localities than the ER may leave this "sorting compartment" for the cis-Golgi by default.

H 015 RETENTION OF MUTANT GLYCOPROTEINS IN THE ER: EVIDENCE FOR BIP INVOLVEMENT WITH ONLY ONE MUTANT CLASS. John Rose, Panos Zagouras, Robert Doms, David Bole, Ari Helenius, and Carolyn Machamer, Departments of Pathology and Cell Biology, Yale University School of Medicine, New Haven, CT. 06510.

In order to understand the basis for selective retention of proteins in the endoplasmic reticulum (ER), we have investigated a panel of mutant vesicular stomatitis virus glycoproteins with structural alterations in the extracellular, transmembrane, or cytoplasmic domains. Analysis of the interaction of heavy chain binding protein (BiP, GRP78) with the newly synthesized wild-type glycoprotein showed that BiP recognized only incompletely disulfide bonded precursors of the glycoprotein and was released once the correct disulfide bonds had formed. Mutations in the extracellular domain which caused protein retention in the ER also caused glycoprotein aggregation, failure to acheive correct intrachain disulfide bonding, and formation of a stable complex with BiP. In contrast, a mutation in the transmembrane domain which leads to glycoprotein aggregation and retention, did not cause incorrect disulfide bond formation and BiP was not retained in the aggregate. A third group of mutations in the cytoplasmic domain resulted in slowed or blocked transport from the ER. These mutations did not detectably affect folding, correct disulfide bonding, or oligomerization of the extracellular domain, nor did they lead to stable complex formation with BiP. We conclude that BiP may be involved in retention of certain misfolded proteins, but that binding by BiP cannot explain retention or slow export of other mutant proteins.

We have also investigated formation of mixed oligomers containing subunits of both wildtype and mutant glycoproteins. When a mutant with a cytoplasmic tail that causes retention in the ER (but which also forms trimers) is expressed in the same cell as the wild-type protein, little or no mixing to form heterotrimers occurs. However, when export of both proteins from the ER is blocked, the wild-type and mutant proteins form heterotrimers efficiently. These results suggest that the wild-type protein is normally physically separated from the mutant protein during trimer formation. Models that might explain this finding will be discussed.

Sorting in the Exocytotic Pathway

H 016 BIOGENESIS OF SECRETORY GRANULES IN VIVO AND IN VITRO Wieland B. Huttner, Hans-Hermann Gerdes, Patrizia Rosa and Sharon A. Tooze
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The secretogranins (chromogranins) are a family of secretory proteins which are widespread constituents of the matrix of secretory granules in endocrine and neuronal cells. We use the secretogranins as model proteins to study the sorting of regulated secretory proteins and the biogenesis of secretory granules in neuroendocrine cells.

The secretogranins contain sorting information to secretory granules which is sufficient to divert a constitutive secretory protein. An immunocomplex consisting of newly synthesized secretogranin I and a monoclonal anti-secretogranin I antibody was efficiently packaged into secretory granules of PC12 cells. To identify this sorting information, we have determined and compared the sequences of chromogranin A, secretogranin I and secretogranin II. Striking common features include a conserved ten-amino-acid motif, abundance of acidic residues, and an alternating helix-turn secondary structure. In line with the latter two properties, the secretogranins have been found to aggregate in the presence of calcium. The secretogranin aggregates include other regulated secretory proteins but exclude constitutive secretory proteins. The calcium-induced aggregation of secretogranins in vitro is much reduced at neutral pH, and the packaging of secretogranins into secretory granules in vivo is inhibited by ammonium chloride. These results suggest that an aggregation of the secretogranins may be the first step in their sorting from constitutive secretory proteins.

To further elucidate the mechanism of secretory granule formation, we have identified the first intermediate in this process, the immature secretory granule, in PC12 cells and demonstrate the sorting of a secretogranin II to immature secretory granules in a cell-free system. The formation of immature secretory granules was as fast ($t_{1/2}$ ≈ 5 min) as that of constitutive secretory vesicles identified by the presence of a rapidly secreted heparan sulfate proteoglycan. Using the cell-free system, the sorting of secretogranin II and the proteoglycan into newly formed immature secretory granules and constitutive secretory vesicles, respectively, was found to occur directly upon exit from the trans Golgi network. The cell-free formation of immature secretory granules, like that of constitutive secretory vesicles, was dependent on ATP. Interestingly, the formation of both types of post-Golgi secretory vesicles was inhibited by GTPγS, suggesting that the budding of secretory vesicles from the trans Golgi network involves GTP-binding proteins.

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H 017 MEMBRANE PROTEIN SORTING IN ENDOCRINE CELLS, Regis B. Kelly, Lois Clift-O'Grady, Anson Lowe, Adam Linstedt and Eric Grote. Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448

Synaptic vesicles are easily isolated because of their uniform size and they contain unique membrane proteins. In contrast to nervous tissue, endocrine cell lines provide a tractable system for the study of synaptic vesicle biogenesis. Endocrine cell lines express the known synaptic vesicle proteins and package them into a vesicle that has a size, density and protein composition very similar to those of authentic brain synaptic vesicles. In the endocrine cell line, PC12, the synaptic vesicle-like structures are generated by endocytosis, as are authentic synaptic vesicles. Since the PC12 vesicles exclude known markers of receptor-mediated endocytosis we suggest that they arise by a novel endocytotic pathway. Chimeric proteins are being constructed to identify sorting domains. When a synaptic vesicle protein is expressed stably or transiently in non-neuronal cell lines, it is found in vesicles dissimilar to synaptic vesicles. We suggest that a synaptic vesicle protein is targeted to pre-existing endocytotic structures in non-neuronal cells, but in endocrine cells expressing all the synaptic vesicle proteins, a novel pathway arises that generates synaptic vesicle structures.

H 018 SORTING OF CELL SURFACE GLYCOPROTEINS IN THE TRANS GOLGI NETWORK OF MDCK CELLS, Kai Simons, Angela Wandinger-Ness and Mark K. Bennett, EMBL, Postfach10.2209, D-6900 Heidelberg, Fed. Rep. Germany.

The simple epithelia, lining the body cavities form cellular sheets between the external and internal environment. In order to perform their boundary function, epithelial cells have evolved a very specialized cellular architecture. The plasma membrane of each epithelial cell is divided into two domains with distinct protein and lipid compositions. We are using the MDCK cell line as our experimental model to study epithelial polarity. Using cells, grown on permeable supports, an epithelial sheet is obtained that closely mimics the organization seen *in vivo*. Three membrane transport markers--an apical protein (influenza virus hemagglutinin), a basolateral protein (vesicular stomatitis virus G protein), and a membrane lipid marker (C6-NBD-ceramide), have been used to study how proteins and lipids are transported and sorted to the two cell surface domains. Sorting has been localized to the trans-Golgi network. Apical and basolateral components are segregated into separate carrier vesicles. Conditions have been established that lead to the formation of both apical and basolateral carrier vesicles from the trans-Golgi network and to their release from the cells (1). The vesicles have well-defined sedimentation properties and the orientation expected of transport vesicles and they have been isolated by immunoisolation and characterized. The protein patterns of the apical and basolateral carrier vesicles isolated from virus-infected MDCK cells have been compared by 2-D gel electrophoresis. The results show that the vesicles have proteins in common as well as proteins presumably specific for the apical and the basolateral routes. Studies are in progress to define whether the mechanisms for both apical and basolateral sorting in the trans-Golgi network are specific for epithelial cells or whether one of the pathways has a counterpart in non-polarized cells such as fibroblasts (2).

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Genetic and in Vitro Analysis of Cell Compartmentalization

Mechanisms of Nuclear Assembly

H 019 TRANSLOCATION THROUGH THE YEAST NUCLEAR ENVELOPE, Michael G. Douglas, Jerry Allen, Alvrom Caplan, Department of Biochemistry and Biophysics, University of NC at Chapel Hill School of Medicine, Chapel Hill, NC 27599

Protein translocation from the cytoplasm to the nucleus is a signal directed event which is poorly understood. Little is known regarding the molecular events involved in signal recognition and the apparatus which mediates translocation. In order to open nuclear protein transport to molecular genetic analysis, we have initiated a biochemical approach in yeast Saccharomyces cerevisiae to isolate the nuclear pore complex. When yeast nuclei are sequentially extracted with detergent nucleases and salt they exhibit a nuclear pore-matrix lamina (PML) fraction like that in higher eukaryotes. The nuclear pore itself appears to organize 10-nm filaments into an assembly which exhibits an eight-fold rotational symmetry. SDS gel analysis of this fraction reveals ~12 prominent proteins. Polyclonal antisera to these proteins have been prepared in rabbits and used in three ways. First, we have determined the subnuclear localization patterns on immunofluorescence. Second, we have determined that some will block the in vitro transport of proteins who isolated yeast nuclei. Third, we have used antisera to screen a λgt11 expression library to select the cloning DNA. Data on these will be discussed.

In related studies, yeast nuclei rapidly purified using a cytochalasin B technique retain transport properties comparable to nuclei in situ. Radiolabeled proteins prepared in a translation lysate bind to nuclei and are subsequently transported to a protected site by a process which requires ATP and Ca^{2+}. This translocation assay has been used in conjunction with specific antibodies to define PML proteins which are involved in translocation. These are the antisera which have been used to screen an expression library. Sequence analysis of some of these will be discussed. These studies provide the basis for genetic analysis of MPL function. Supported by UPS Grant GM41758.

H 020 ANALYSIS OF NUCLEAR PROTEIN IMPORT: CHARACTERIZATION OF A PUTATIVE TRANSPORT RECEPTOR, Stephen Adam and Larry Gerace, Dept. of Molecular Biology, Scripps Research Institute, La Jolla, CA 92037

Import of most proteins into the nucleus is thought to occur by mediated transport across the nuclear pore complex, and is directed by short amino acid stretches within nuclear proteins called nuclear location sequences. Presently, little is known about how these sequences function. We have performed binding and crosslinking studies with synthetic peptides to analyze cellular proteins that interact with the nuclear location sequence (pro-lys-lys-lys-arg-lys-val) of the the SV40 T antigen (Adam et al, 1989, Nature 337:276-279). This work identified one major cellular polypeptide that binds this sequence with the specificity and high affinity expected for a transport receptor. This protein is present in cytosol, nuclear envelope, and nuclear content fractions of cells, suggesting that it may be a carrier that cycles between cytoplasm and nucleus during the process of nuclear protein import. We recently isolated this protein from rat liver nuclear envelopes using column chromatography, and are using antibodies to the protein to analyze its functions in a cell-free nuclear protein import system that we recently devised. This system involves digitonin-permeabilized cultured mammalian cells, whose nuclei (which are functionally intact) strongly accumulate fluorescently-labelled proteins containing nuclear location sequences from the surrounding medium when the permeabilized cells are incubated at 33°C in the presence of ATP and cytosol.

H 021 GENETIC AND BIOCHEMICAL ANALYSIS OF NUCLEAR ASSEMBLY IN YEAST, Pamela Silver, Annette Chiang, Mark Osborne, Takao Kurihara and Ursula Stochaj, Department of Biology, Princeton University, Princeton, NJ 08544

Yeast mutants have been characterized that exhibit a defect in nuclear protein localization (termed *npl* for nuclear protein localization). To isolate these mutants, we employed chimeric proteins consisting of nuclear localization sequences (NLS) fused to the precursor form of the mitochondrial proteins cytochrome c_1 and $F_1\beta$ATPase. A yeast strain deficient in cytochrome c_1 (*cyt1*) or $F_1\beta$ATPase (*atp2*) is unable to grow on nonfermentable carbon sources, such as glycerol. Yeast strains producing NLS-cytochrome c_1 or NLS-$F_1\beta$ATPase cannot complement the corresponding mutant strain for growth on glycerol because the mitochondrial proteins are preferentially localized to the nucleus. Thus, in these fusion proteins the functional NLS can act before the mitochondrial signal sequence. By selecting for growth on glycerol at 30°C and temperature-sensitve growth at 36°C, we have isolated mutants that affect nuclear protein localization. Thus far, two complementation groups have been identified.

The *NPL1* and *NPL2* genes have been cloned. The *NPL1* DNA sequence contains a 1989 base pair open reading frame encoding the *NPL1* gene product. Three potential membrane-spanning regions are found within the first 240 amino-terminal residues. A sequence sharing 43% amino acid identity and many conservative substitutions with the amino terminus of the *E. coli* DnaJ protein is located between the second and third hydrophobic regions. An anti-*NPL1* antibody has been used to localize the protein by immunofluorescence to the nuclear envelope and to analyze its possible orientations in the membrane. *NPL1* is allelic to *SEC63*, a yeast gene important for assembly of proteins into the ER.

We have developed an *in vitro* nuclear localization reaction with components derived from yeast. For substrate, we employ human serum albumin (HSA) to which peptides corresponding to different nuclear localization sequences have been chemically cross-linked. Isolated yeast nuclei supplemented with an ATP-regenerating system specifically bind NLS-HSA conjugates. The binding is saturable and HSA with a mutated NLS binds with 100-fold reduced affinity. We have identified yeast proteins of 70 and 59kD that bind nuclear localization peptides of SV40 T-antigen, *Xenopus* nucleoplasmin, and the yeast proteins Gal4 and histone H2B. Some of these binding proteins fractionate with nuclei, are extractable with salt, but not detergent and have the properties expected for a receptor that would act to direct proteins to the nucleus. Reconstitution experiments suggest that salt-extracted NLS-binding proteins can be added back to trypsin-treated nuclei to restore the first step in nuclear protein import *in vitro*.

Endomembrane Traffic-I

H 022 ROLE OF GTP IN REGULATION OF VESICULAR TRANSPORT, William E. Balch, Steven N. Pind, R. Schwaninger, and Helen Plutner, Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037. Transport of protein between the ER and the Golgi, and between Golgi compartments is regulated by GTP hydrolysis and members of a new family of *ras*-related low molecular weight GTP-binding proteins, the *rab* gene family. To study the regulation of transport by GTP a series of peptides were synthesized against putative functional domains of the *rab* proteins. Several peptides encompassing the *rab* protein effector domain were found to be potent inhibitors of both ER to Golgi and intra-Golgi transport *in vitro*. Analysis of the kinetics of inhibition of transport by peptide analogues revealed that transport is rapidly inhibited ($t_{1/2}$ = 30 sec). The site of inhibition of ER to Golgi transport was found to be a late step in transport, immediately following a Ca^{2+}-dependent step involved in the fusion of transport vesicles with the *cis* Golgi compartment. Additional studies strongly promote a model for regulation of transport in which a homologous protein to *ras* -GAP (to be referred to as *rab*-GAP) is the target of the peptide analogue inhibition of transport and is an essential general component in the regulation of GTP hydrolysis and the delivery (fusion) of carrier vesicles to the *cis* and *medial* Golgi compartments.

H 023 NEW VIEWS OF MEMBRANE DYNAMICS BETWEEN THE ER AND THE GOLGI. Richard D. Klausner, Julie G. Donaldson, Hans-Peter Hauri*, Lydia C. Yuan and Jennifer Lippincott-Schwartz, Cell Biology and Metabolism Branch, NICHD, National Institutes of Health, Bethesda, MD, and *Biozentrum, Basel, Switzerland.

A variety of pieces of evidence have pointed to an increasing complexity of structure and function of the organellar system that precedes the Golgi in the secretory pathway. These involve the regulation of transport, degradation and the possibility of the retrieval of proteins into the ER. In studying the effects of the fungal anti-metabolite, Brefeldin A (BFA), we noted an intriguing phenotype that underlay the previously reported inhibition of secretion from the ER seen with this drug. Within minutes of the addition of BFA, the Golgi cisternae were no longer apparent in the cell and proteins contained within the cis/medial and trans Golgi cisternae were now found in the endoplasmic reticulum. In order to determine whether this retrograde movement had a counterpart in the normal cell, we examined the details of the pathway by which Golgi proteins were returned to the ER. Within two minutes after the addition of BFA, the Golgi cisternae swelled and gave rise to long "necklace-like", tubulo-vesicular structures. These structures were associated with microtubules. Golgi proteins appeared transiently in these "necklaces" before redistributing into the ER. The formation of these necklaces could be inhibited by reducing temperatures to below 16°C. The "necklace" structures were not observed when cells were preincubated with drugs that depolymerize microtubules or that deplete cellular ATP. Although there is no detectable Golgi structure in the presence of BFA, examination of the distribution of a 53 kD component of intermediate vesicles (developed by Hans-Peter Hauri) situated between the ER and Golgi revealed that these vesicles remained distinct from the ER over long periods of time in the presence of BFA. When cells were treated with BFA to allow Golgi proteins to redistribute into the ER and then treated with microtubule disrupting agents, the Golgi proteins exited the ER and accumulated in large structures that co-localized with the 53 kD marker. Indiginous resident ER proteins did not leave the ER in the presence of BFA and nocodazole. These findings suggest that in the presence of BFA a constant cycling of membrane occurs between the ER and an intermediate compartment, and that the retrograde arm of this cycle is blocked by nocodazole. By lowering the temperature to 16°C in the absence of drugs, the 53 kD intermediate compartment marker accumulated in a Golgi-like structure and in necklace-like extensions that were indistinguishable from those first observed in the presence of BFA. These necklaces were abolished by dropping the temperature below 16°C or by treatment with nocodazole. In the absence of BFA, these necklaces only contained the 53 kD marker and no Golgi proteins. However, upon addition of BFA, the necklaces could now be co-stained with the 53 kD protein and Golgi markers. The implications of these findings for an ER recycling pathway will be discussed.

H 024 A MOLECULAR DISSECTION OF VESICULAR TRANSPORT, James E.Rothman, Department of Biology, Lewis Thomas Laboratory, Princeton University, Princeton, New Jersey 08544-1014.

Protein transport between successive cisternae of the Golgi stack is reconstituted when isolated Golgi membranes are incubated with cytosol and ATP. This transport is due to rounds of budding and fusion of coated vesicles. When transport is blocked by adding the nonhydrolyzable analogue of GTP, GTPγS coated vesicles massively accumulate. The coated vesicles can be purified by density gradient centrification following extraction with salt, and contain a number of characteristic polypeptide chains including several GTP-binding proteins, but not clathrin. Following transfer of budded coated vesicles between cisternae, the coats are removed, and fusion proceeds in an ATP dependent process. Fusion requires an *NEM-sensitive protein (NSF)*, a series of soluble *NSF attachment proteins (SNAPS)*, palmityl-CoA as well as other cytosolic factors.

H 025 MOLECULAR REQUIREMENTS FOR VESICLE BUDDING AND FUSION EARLY IN THE SECRETORY PATHWAY, Randy Schekman, David Baker, Christophe D'Enfert, Linda Hicke, Midori Hosobuchi, Chris Kaiser, Nancy Pryer, and Michael Rexach, Division of Biochemistry and Molecular Biology, University of California, Berkeley, CA 94720

Two stages in vesicular transport of secretory proteins from the endoplasmic reticulum to the Golgi apparatus have been defined by analysis of sec mutant yeast cells. One group of mutants (sec12, 13, 16, 23, 21) fails to form the small vesicles that mediate transport, and another group (sec17, 18, 22) forms vesicles that fail to fuse with the Golgi apparatus. Selective genetic interactions among mutations of each group suggest that subsets of gene products act closely together, perhaps as protein complexes, to facilitate vesicle budding or fusion.

Protein transport from the ER to the Golgi complex has been reconstituted in vitro in a reaction that reproduces many of the physiologic processes. Transport requires Sec gene products, cytosol, ATP, Ca^{2+}, Mn^{2+}, and is sensitive to GTPγS. Defective transport is seen in extracts that contain membranes and cytosol prepared from sec12, sec13, sec16, sec18, sec19, sec23, and ypt1-1 mutant cells. Cytosol from wild type cells restores transport in membranes of sec13, sec18, sec19, sec23, and ypt1-1 cells. This biochemical complementation assay has been used to purify a functional form of the Sec23 protein. A complex (300-400 kDa) including Sec23p (85 kDa) and an unidentified 105 kDa polypeptide copurify by conventional chromatographic procedures. When Sec23p is overproduced, biochemical complementation of a sec23 mutant lysate allows the isolation of a functional monomeric form of Sec23p.

Sec23p and other proteins that appear to function in vesicle budding may be recruited to membranes through interaction with Sec12p, a 70 kDa integral membrane glycoprotein with an N-terminal 40 kDa domain that faces the cytosolic surface of the ER or Golgi membrane. The N-terminal domain is essential for the transport function of Sec12p, though the C-terminal lumenal domain is not.

Three stages in transport have been recognized by detection of a vesicular intermediate released early during incubation in the in vitro reaction. A budding step requires cytosol, ATP, and functional forms of Sec12p and Sec23p. Vesicle targeting requires Ypt1p and Sec18p. Reactions deficient in Ypt1p or Sec18p produce vesicles that sediment more slowly than the Golgi compartment. Finally, vesicle fusion requires Ca^{2+}, ATP, and cytosol. Ca^{2+}-deficient incubations produce vesicles that sediment along with the Golgi compartment.

Endomembrane Traffic-II

H 026 CLATHRIN COATED PITS IN VITRO: ASSEMBLY AND DYNAMICS, M.S. Moore, D.T. Mahaffey, J.S. Peeler and R.G.W. Anderson, Department of Cell Biology & Neuroscience, U.T. Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235.

In 1987 we reported on the development of a membrane preparation that supports the assembly of clathrin coated pits. (1). This membrane system has been used to demonstrate that purified coat proteins from bovine coated vesicles will assemble coated pits at a limited number of assembly sites following treatment to remove endogenous clathrin (2). Moreover, when these membranes with their endogenous coated pits are allowed to warm to 37°C, the flat lattices round up and a large percentage of the clathrin dissociates from the membrane (3). This dissociation is inhibited by treatment of the membranes with the ATP destroying enzyme, apyrase. All of these studies have been carried out on membranes that are derived from cells that have been allowed to attach and spread on the surface of a poly-L-lysine coated substratum at 37°C. An alternate method of membrane isolation has been developed in which the cells are attached to the coated coverslip by centrifugation at 4°C. The membranes prepared by this procedure contain several fold less clathrin than membranes from 37°C spread cells; moreover, by rapid-freeze deep-etch microscopy the regions of clathrin-coated membrane that are present resemble much more closely the coated pits present on intact cells (i.e. their morphology ranges from flat to deeply invaginated). Although these coated pits will also become increasingly invaginated when warmed, the coated pits do not disassemble but seem to arrest at the pinching off stage. Studies in progress are designed to determine if pinching off is stimulated by the addition of specific cytoplasmic factors. Additionally, we are also comparing the two types of membranes for their ability to support coated pit assembly. Based on these observations, apparently not all coated pits are created equal.

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H 027 KINETICS OF ENDOSOME FUSION IN INTACT CELLS, Frederick R. Maxfield, Kenneth W. Dunn, Laurence A. Borden, and Nita H. Salzman, Department of Pathology, Columbia University College of Physicians and Surgeons, New York, New York 10032.
Following binding at the cell surface, ligands and receptors can follow several intracellular routes. We have used fluorescent transferrin to follow the receptor recycling pathway and fluorescent LDL or α_2-macroglobulin to follow the pathway to lysosomes. Using image intensification fluorescence microscopy and digital image analysis, we measured the fluorescence intensity of single endosomes in intact cells. We have been able to determine the kinetics of fusion and segregation of endosomes following endocytosis of fluorescent ligands. Vesicles containing newly endocytosed ligands and receptors are delivered to a fusion competent "sorting endosome". Each sorting endosome has a lifetime of approximately 8 minutes during which it exports recycling components while simultaneously fusing with new endosomes and accumulating ligands which are targeted to lysosomes. Thus, LDL accumulates approximately 40-fold during the lifetime of a sorting endosome, but transferrin accumulates only 3-4 fold. The endosomes containing LDL or α_2-macroglobulin become incompetent for fusion with new endosomes with a half time of about 8 minutes. The kinetics of fusion accessibility and maturation to fusion inaccessibility have been confirmed by an independent fluorescence assay based on the mixing of sequentially formed endosomes containing fluorescein labeled ligands chased by anti-fluorescein IgG. The kinetics of vesicle fusion and budding documented in these studies suggest that high efficiency recycling of membrane components could operate as a default pathway as a result of many iterations of a moderately efficient sorting process.

H 028 MOLECULAR ANALYSIS OF PROTEIN SORTING ALONG THE PATHWAY TO LYSOSOMES
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Mannose 6-phosphate receptors (MPRs) carry soluble lysosomal enzymes from the *trans* Golgi network (TGN) to late endosomes and then return to the TGN for another round of lysosomal enzyme transport. We have reconstituted the recycling of the 300kD MPR from late endosomes back to the TGN in a cell-free system[1] and are analyzing the cytosolic components required for this transport step. MPRs use clathrin-coated vesicles for export from the TGN and also during endocytosis. However, it is not yet known whether clathrin mediates MPR recycling back to the TGN. We utilized clathrin heavy chain antibody X19 (generously provided by Dr. F. Brodsky, UCSF) which inhibits endocytosis *in vivo*[2] to test its effect on MPR transport to the TGN. Under conditions in which the *in vitro* endocytosis of transferrin (assayed according to Smythe *et al.*[3]) was inhibited ~60%, X19 IgG had no effect on MPR recycling to the TGN. Thus MPR recycling does not appear to involve clathrin. In addition, the clathrin dependence of *in vitro* endocytosis documents the physiological significance of this reaction. We have also identified a novel, NEM-sensitive cytosolic factor (factor Y) required for MPR recycling, that is distinct from NSF, a protein required for ER→Golgi and intra-Golgi transport, as well as endocytic vesicle fusion. Factor Y is entirely cytosolic and is proteinaceous as shown by its sensitivity to trypsin or boiling; it has an apparent Mr of ~150K by gel filtration and velocity sedimentation. Cytosol depleted of NSF activity fully restores MPR recycling *in vitro*. In contrast to NSF, factor Y may only be involved in a limited number of transport events, and its characterization will enhance our understanding of the molecular mechanisms underlying vesicular transport.

Unlike most receptors, 300kD MPRs are localized primarily in the TGN and in endosomes, and they cycle constitutively between these compartments. Yet, when present at the cell surface, MPRs can undergo conventional receptor-mediated endocytosis. We constructed a chimeric receptor, comprised of EGF receptor extracellular and transmembrane domains joined to the MPR cytoplasmic domain, to test whether the MPR cytoplasmic domain contained sufficient information to direct a cell surface receptor into both of these transport pathways. The expressed protein was stable, bound EGF with high affinity, and was efficiently endocytosed and recycled to the cell surface, ± EGF. If the cytoplasmic domain alone is responsible for sorting native MPRs, chimeric receptors might have been expected to be located primarily in the TGN and in endosomes at steady state. Surprisingly, while most endogenous MPRs were intracellular, >85% of the chimeric receptors were located at the cell surface. These experiments demonstrate that the MPR cytoplasmic domain is not sufficient to alter the distribution of the EGF receptor, and suggest a role for extracellular and transmembrane domains in MPR routing.

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H 029 BIOCHEMICAL CHARACTERIZATION OF THE EARLY EVENTS IN RECEPTOR-MEDIATED ENDOCYTOSIS, Sandra L. Schmid, Elizabeth A. Smythe, Thomas. E. Redelmeier and Laura L. Carter, Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA, 92037.

We have developed separate cell-free assay systems which measure two distinct early events in receptor-mediated endocytosis in order to begin their biochemical characterization. One assay measures coated pit formation and the selective packaging of membrane proteins and receptors into coated vesicles *in vitro*. In it, we take advantage of a novel cell-free system involving 'semi-intact' cells: cells in which a portion of the plasma membrane has been removed so as to deplete them of cytoplasm but otherwise maintain their intracellular organelles intact and accessible to exogenously added reagents. Our approach is as follows: radiolabelled ligands (usually diferric transferrin) are bound to intact cell monolayers on ice, unbound ligand is removed and the cells are made semi-intact. The semi-intact cells are washed free of cytosol and reincubated at 37°C with or without added cytosol and an ATP regenerating system. Following incubation, internalization of prebound ligands into a sealed membrane-bound endocytic compartment is determined. We have developed a novel reagent for this purpose which involves biotinylation of a protein ligand such as transferrin (Tfn) using the cleavable biotinylating reagent, NHS-SS-Biotin (Pierce). Biotinylated-^{125}I-Tfn is determined by adsorption to avidin-Sepharose. Ligand internalization is assessed by acquisition of resistance to cleavage by the membrane impermeant sulfhydryl reagent, glutathione. Using this assay, we find that prebound Biotin-S-S-^{125}I-Tfn (^{125}I-BSST) is rapidly internalized into a glutathione-resistant sealed membrane compartment with kinetics comparable to those observed in intact cells. Acquisition of glutathione resistance is dependent on ATP, cytosol and incubation at elevated temperatures. We are currently using this assay to fractionate and identify the cytosollic requirements. A second assay measures the specific membrane fusion event between primary endocytic (coated) vesicles and 'early' endosomes. Membrane fusion is assessed in a manner similar to that described by W.A. Braell (1987, Proc. Natl. Acad. Sci. (USA) **84**:1137-1141), by measuring the mixing of vesicle contents between 'donor' coated vesicles containing avidin-HRP and an 'acceptor' fraction of early endosomes containing biotinylated-transferrin . Highly purified coated vesicle 'donors' and enriched endosomal 'acceptors' containing the appropriate endocytic tracers are obtained from perfused rat liver. This assay will be used to study membrane recognition and fusion using highly purified vesicle fractions in order to analyze the specificity of these fusion events and to identify the protein components which regulate and mediate these membrane interactions.

Cytoskeletal Involvement in Cellular Compartmentalization

H 030 ROLE OF MICROTUBULES IN INTRACELLULAR TRANSPORT, Thomas E. Kreis, Rainer Duden, Dagmar Hennig, Wai Chang Ho, Paula Karecla, Janet E. Rickard, Jochen Scheel and Peter van der Sluijs, EMBL, Meyerhofstr. 1, Postfach 10.2209, D-6900 Heidelberg, Fed. Rep. Germany.
The Golgi apparatus, as well as late endosomes and lysosomes, colocalizes in the perinuclear region around the microtubule organizing center (MTOC) in interphase fibroblasts. This spatial apposition of key organelles involved in intracellular membrane traffic may facilitate intercompartmental transport and connect the secretory and endocytic pathways. Clustering of Golgi elements and endosomes around the MTOC depends on microtubules. Disruption of microtubules by nocodazole leads to random scattering of Golgi elements and endosomes throughout the cytoplasm, similar to dispersal of these organelles at the onset of mitosis. Removal of nocodazole and subsequent microtubule repolymerization leads to reclustering of these organelles around the MTOC within 30-60min. This process has been characterized in living cells using vital staining and video-enhanced fluorescence microscopy. Reclustering occurs retrogradely along microtubules and is independent of intermediate filaments and microfilaments.

We have partially purified kinesin and cytoplasmic dynein from HeLa cells. Furthermore, we have identified a novel nucleotide-sensitive microtubule binding protein of M_r 170,000. These microtubule-based motor proteins and the 170K protein may be involved in the directed movement of cellular organelles along microtubules. In addition we are in the process of characterizing the function of a 110K protein on the molecular level. This protein is associated with the cytoplasmic face of Golgi membranes and interacts with microtubules *in vitro* (1). It is present in *Xenopus laevis* eggs, where it is hyperphosphorylated in meiosis. We are currently investigating whether this cell-cycle-dependent hyperphosphorylation of the 110K protein plays a part in regulating the Golgi-microtubule interaction or in disrupting the Golgi apparatus at the onset of mitosis.

We have established quantitative *in vitro* binding assays to investigate interactions between microtubules and endosomes, Golgi elements or Golgi-derived exocytic carrier vesicles. These assays are being used to identify and characterize the cytosolic, organellar membrane and microtubule binding components involved in the interaction of the specific organelles with microtubules.

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H 031 MYOSIN-I BINDS TO CELLULAR MEMBRANES AND POWERS THEIR MOVEMENTS ALONG ACTIN FILAMENTS, Thomas D. Pollard, Henry Zot, Steve Doberstein and Shigenobu Yonemura, Department of Cell Biology and Anatomy, Johns Hopkins Medical School, Baltimore, MD 21205.

Myosin-I is a class of single-headed myosin originally discovered in Acanthamoeba and now known to be widely distributed in nature. Others have shown that cells contain multiple myosin-I isozymes. The known myosin-I's have a typical myosin head and a C-terminal domain of variable length (work of Hammer and colleagues). Purified myosin-I from Acanthamoeba binds to cellular membranes stripped of all peripheral proteins with a K_d of 140nM and a stoichiometry exceeding that of any single membrane protein (Adams and Pollard, Nature, August 1989). Salt stripped plasma membranes also bind myosin-I (Miyata et al, JCB, Oct 1989) Anionic lipids are one of the membrane binding sites, since myosin-I also binds to purified lipid vesicles. Experiments with proteolytic fragments of myosin-I suggested that the membrane binding site is in a 30kDa domain distal to the head in the sequence. This has been confirmed by showing that a fusion protein containing this part of myosin-I also binds to stripped membranes. Deletion and mutagenesis of this domain should localize the binding site more precisely. After binding myosin-I, isolated stripped membranes can translocate along bundles of actin filaments in the presence of ATP. Some of these movements occur in bursts, some are continuous over 50 um at high velocities >3 um/s. By immunofluorescence with monoclonal antibodies, myosin-I is concentrated on various membranes in Acanthamoeba. One antibody, M1.1, stains the contractile vacuole very strongly. Other antibodies stain the plasma membrane and small particles throughout the cytoplasm. These observations suggest that the various myosin-I isozymes are targeted to specific membranes or organelles, most likely by specific receptors. The unspecific affinity of myosin-I for phospholipids may provide a mechanism to concentrate myosin-I on the cytoplasmic face of membranes. This together with the reduction in dimensionality would favor associations of mysoin-I with even low affinity receptors on specific membrane compartments. These new findings provide the biochemical basis for intracellular membrane traffic along actin filaments and a possible mechanism for plasma membrane movements including ruffling and pseudopod extension.

Genetic and in Vitro Analysis of Cell Compartmentalization

Preprotein Targeting, Translocation and Processing

H 100 MAPPING OF THE SIGNAL SEQUENCE BINDING SITE ON SRP 54. DIETER ZOPF, HARRIS D. BERNSTEIN AND PETER WALTER, DEPARTMENT OF BIOCHEMISTRY AND BIOPHYSICS, UNIVERSITY OF CALIFORNIA, MEDICAL SCHOOL, SAN FRANCISCO, CA. 94143-0448
The 54K subunit of the signal recognition particle (SRP) is responsible for signal sequence recognition. Crosslinking experiments have shown that the signal peptide is in direct contact with SRP54 (Krieg et al. 1986, PNAS 83, 8604-8608; Kurzchalia et al. 1986, Nature 320, 634-636). SRP54 which has recently been cloned (Bernstein et al. 1989, Nature 340, 482-486; Römisch et al. 1989, ibid., 478-482) contains a putative GTP binding site and a Met-rich region at the carboxy-terminus. Based on secondary structure prediction the latter region has been suggested to form a binding pocket for signal peptides. In order to test this hypothesis we have crosslinked the signal peptide of preprolactin to SRP in vitro in a wheat germ translation system. Crosslinked SRP was released from the ribosomes, sucrose gradient purified and subsequently digested with elastase. Two major digestion products of about 32K and 27K were obtained. In order to localize these peptides within the primary structure of SRP54 we currently trying to immunoprecipitate them with a variety of antibodies against SRP54. For this purpose we have generated antibodies specific for the N-terminus and the C-terminus of SRP54 that should allow us to map the crosslink site.

H 101 ISOLATION AND CHARACTERIZATION AND OF A RIBOSOME BINDING PROTEIN FROM ENDOPLASMIC RETICULUM, Adam J. Savitz and David I. Meyer, Dept. of Biological Chemistry and the Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA 90024. In eukaryotic cells, secretory proteins are synthesized on ribosomes attached to the endoplasmic reticulum (ER). After signal recognition particle (SRP) recognizes and directs the polysomes synthesizing secretory proteins to docking protein in the ER, the polysomes become attached to the ER membrane by a protein-mediated mechanism. The assay system that has been used to study ribosome binding consists of initially stripping rough microsomes of their ribosomes with puromycin, nuclease, and high salt, and then adding labeled ribosomes to the stripped rough microsomes (RMsn) in low salt buffer. To begin to identify the proteins necessary for translocation after the SRP mediated step, controlled proteolysis was used to eliminate the ribosome binding to the ER. Gentle proteolysis with certain proteases eliminated only a small fraction of the ribosome binding activity but generated proteolytic fragments that inhibited the binding of ribosomes to RMsn that had not been proteolyzed. As visualized on silver-stained SDS-acrylamide gels, the initial active protease digest contained approximately 40 protein species. Column chromatography was used to separate this digest. In low salt and at neutral pH, the activity could be bound to both anionic resins (CM, SP, heparin agarose, and phosphocellulose) and cationic resins (QAE and DEAE). In addition, an affinity column was made by attaching salt-washed canine pancreatic ribosomes to Affigel-10. At present, through the combination of various columns, the purest active fraction contains four fragments. Once the fragment with activity is determined, anti-fragment antibodies will then be used in order to identify the entire protein from which the fragment is derived, to try to inhibit ribosome binding as well as *in vitro* protein translocation, and to clone the cDNA encoding this protein for further structural and functional analysis.

H 102 ASSEMBLY OF TRANSLOCATION COMPETENT VESICLES FROM DETERGENT SOLUBILIZED COMPONENTS. Christopher Nicchitta, Giovanni Migliaccio and Günter Blobel, Laboratory of Cell Biology, Howard Hughes Institute, Rockefeller University, New York, NY 10021. Salt and EDTA washed canine pancreas rough microsomes were solubilized with detergent, the mixture centrifuged to remove components sedimenting at S values greater than 50 S and vesicles reassembled by dialysis of the high speed supernatant. In in vitro translation/translocation assays the reconstituted vesicles processed preprolactin in a cotranslational, SRP dependent manner. Mature prolactin was inextractable with EDTA or high salt, sedimented with the membrane fraction and was protected from digestion with exogenous protease. These results indicate that the reconstituted vesicles were competent for the targeting and translocation of secretory precursors. The reconstituted vesicles were devoid of lumenal proteins including BiP and PDI, suggesting that these proteins are not absolutely required for translocation of secretory precursors. A number of rough ER localized glycoproteins have been implicated in the processes associated with nascent chain targeting and translocation. To attempt to determine the glycoprotein requirement for these events, we have initiated studies on vesicles assembled from detergent extracts depleted of glycoproteins by chromatography on Con-A Sepharose columns. The results of these studies will be presented.

H 103 PROTEINS IN THE VICINITY OF MICROSOMAL MEMBRANE BOUND RIBOSOMES AS DETECTED BY A REVERSIBLE CROSSLINKER. Paula G. Collins and Reid Gilmore. Department of Biochemistry, University of Massachusetts Medical Center, Worcester, MA. 01655
One of our main research interests is the identification of integral membrane proteins of the mammalian endoplasmic reticulum that are involved in protein translocation. We are currently using membrane-bound ribosomes to guide us to these proteins. We have developed an assay in which the water soluble, membrane impermeable, thiol-cleavable, crosslinker 3,3'-dithio*bis* (sulfosuccinimidylpropionate)(DTSSP) was added to rough microsomal membranes under non-reducing conditions. The membrane was later solubilized with a nonionic detergent and layered over a sucrose cushion. The centrifugation conditions were such that the ribosomes and any crosslinked proteins would selectively pellet. This pellet was then resuspended under reducing conditions to reverse the crosslinks. The ribosomes were then pelleted again, leaving any previously attached proteins in the supernatant. A specific group of proteins were reproducibly crosslinked to the ribosome with this crosslinking reagent. One protein has been identified by Western blotting as the 35K subunit of the signal sequence receptor (SSR). Further analysis of these proteins has been done by blotting with concanavalin A, Western blotting with antibodies to the signal recognition particle receptor and antibodies to ribophorin I. We have also analyzed these proteins with these techniques after the crosslinked, solubilized proteins were separated by sucrose gradient ultracentrifugation. We propose that the proteins identified by this procedure are components or nearest neighbors of the translocation apparatus.

H 104 PORTIONS OF THE MITOCHONDRIAL PROTEIN IMPORT SIGNAL OF THE F_1-ATPASE β SUBUNIT PRECURSOR ACT TO MAINTAIN AN IMPORT COMPETENT CONFORMATION. David M. Bedwell, University of Alabama, Birmingham, AL 35294.
It is generally accepted that mitochondrial precursor proteins must attain a loosely folded conformation prior to translocation across the mitochondrial membranes. Cytosolic factors such as the HSP70 family of stress induced proteins have been implicated in the maintenance of this conformation. It has previously been shown that the F_1-ATPase β subunit precursor contains redundant targeting information [MCB 7:4038 (1987)]. In order to determine the possible role of these redundant sequences in the maintenance of import competence prior to translocation, I have examined the ability of a series of mutant targeting signals of the F_1-ATPase β subunit precursor to assist in the maintenance of an import competent conformation. Results from both in vivo and in vitro experiments indicate that the redundant aspect of the import signal is directly involved in maintaining the import competence of this precursor.

H 105 **Localization of a Matrix Targeting Domain in the Mitochondrial Intermembrane Space Precursor Protein, Cytochrome C Peroxidase,** Denise Ekberg and Jim Kaput, Department of Biochemistry, University of Illinois at Champaign-Urbana, Urbana, Illinois 61801.

Cytochrome *c* peroxidase (CCP) is a hemeprotein located in the intermembrane space of mitochondria in the yeast *Saccharomyces cerevisiae*. Recent results from our lab show that the *in vitro* import of ^{35}S labelled pre-CCP-pro_{175} (a point mutant of preCCP) does not require an electrochemical potential across the inner membrane for processing and compartmentalization, the signal protease maturation step is not chelator sensitive, and the precursor is not processed by a matrix-localized protease. Our interpretation of these results is that preCCP-pro_{175} import is different apparently from the pathways described for Rieske Fe/S protein, cytochrome c_1 and cytochrome b_2. We have constructed signal sequence deletion mutants which have various degrees of the 3' end of the signal sequence deleted. These mutant proteins test whether preCCP-pro_{175} possesses a matrix targetting domain which functions in the absence of its IMS sorting domain. These signal sequence mutants are targeted to the mitochondrial matrix and are sensitive to the ionophore, valinomycin, indicating a requirement for a chemical potential across the inner membrane. The signal mutants are also not processed by the matrix localized protease suggesting that this protein does not require matrix interactions for wild type localization.

H 106 **PROTEINS INVOLVED IN TRANSLOCATION OF MITOCHONDRIAL PRECURSOR PROTEINS**, David B. Miklos, Ann West, Deborah Clark, and Arthur Horwich, Dept. of Human Genetics, Yale University School of Medicine. Mitochondrial precursor proteins are imported from the cytosol through contact sites between inner and outer membranes. We have taken a genetic approach in Saccharomyces to identify components of the translocation machinery, based on an observation that a temperature sensitive lethal mutant affecting the processing enhancing protein (PEP), is defective not only in proteolytic processing but also in translocation. Suppression of both the growth and translocation phenotypes of this mutant, mif1, was accomplished by transformation with high copy plasmids. Two such suppressors of mif1, Smf1&2, are predicted to be extremely hydrophobic and are related to each other. Deletion of both Smf genes severely impairs growth, and mitochondria from the double knockout strain are defective in translocation of precursor proteins. We have recently identified an additional strongly suppressing Smf plasmid, Smf3, that encodes a more hydrophilic protein. Remarkably, when the sequence of this predicted product was compared with known sequences, it identified an ORF flanking a known gene, and exhibited 70% amino acid identity to that predicted protein. The gene, provisionally Smf4, was isolated, and its sequence is being completed. Antibodies are being generated, and deletion experiments are in progress.

H 107 EVIDENCE THAT THE YEAST MITOCHONDRIAL RESPIRATORY CHAIN IS ASSEMBLED THROUGH A COMMON NUCLEATION COMPLEX, Bernard L. Trumpower and Mark Schmitt, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756

Subunit 6 of the mitochondrial cytochrome bc_1 complex is unusual in that it contains an acidic presequence. A yeast mutant (MES5) in which QCR6, the nuclear gene encoding subunit 6, was disrupted with the URA3 gene is petite and lacks the entire cytochrome containing portion of the respiratory chain and the associated cytochrome c reductase and cytochrome c oxidase activities. The URA3 disruption of QCR6 is capable of replacing the acidic presequence of subunit 6 with a positively charged, generic mitochondrial targeting presequence. The MES5 petite strain reverts, is complemented by a plasmid encoded copy of QCR6, and gives rise to a respiratory competent diploid when mated against a rho^0 strain. Mitochondrial protein synthesis and oligomycin sensitive ATPase activities are unaffected in MES5. We conclude that MES5 is not due to a mutation in the mitochondrial genome (rho^-), and is not due to a global impairment of mitochondrial protein import. We postulate that the cytochrome bc_1 and cytochrome c oxidase complexes are assembled through a common nucleation event, and dissociate to form partially or completely assembled complexes at some point after subunit 6 of the bc_1 complex has been added. According to this model an aberrant copy of subunit 6 interrupts assembly of the two respiratory complexes at a stage where they are part of a common precursor complex.

H 108 **In Vitro Processing of Cytochrome c Peroxidase Precursor**, Jim Kaput and Elizabeth Jewell, Departments of Biochemistry and Cell and Structural Biology, University of Illinois College of Medicine, Urbana, Illinois, 61801.

Cytochrome *c* peroxidase is a 33000-da heme protein located in the intermembrane space (IMS) of *Saccharomyces cerevisiae* mitochondria. This nuclearly encoded gene product is synthesized as a precursor (preCCP) containing 68-amino terminal residues that presumably direct the protein to the mitochondria and sort it to the IMS. We have recently shown that a large percentage of the precursor is processed prior to its complete translocation across the outer mitochondrial membrane (Kaput *et al* [1989] *J. Cell Biol.* 109, 101-112). In addition, processing occurred in the absence of an inner membrane potential. Although several control experiments demonstrated that there was no soluble activity on the outside of the mitochondria and that the outer membrane was intact, preCCP might be processed by some protease contaminant in the mitochondrial preparation or the reticulocyte lysate. To test this possibility, mitochondria isolated from a *PEP4* strain were used in an *in vitro* import assay in the presence and absence of pepstatin A, leupeptin, PMSF, antipain, chymostatin, TLCK, TPCK, Trasylol™, and EDTA. PreCCP was processed by *PEP4* mitochondria in the presence of these protease inhibitors yet the majority of the processed form *was not* associated with the mitochondria pellet. These results support our previous conclusion that the processing and release of preCCP occurs on the normal import pathway. We have also determined that processing of preCCP to its mature-sized form occurs by a protease which requires an intact inner membrane for its activity.

H 109 **Involvement of Proteinaceous Pores in the Import into Mitochondria of Matrix and Intermembrane Space Proteins**, Sandy Kirchner and Jim Kaput, Departments of Biochemistry and Cell and Structural Biology, University of Illinois College of Medicine, Urbana Illinois, 61801.

Protein import into mitochondria is thought to occur through proteinaceous pores that span the inner and outer membrane. We have recently obtained results, however, that suggest that cytochrome c peroxidase (CCP), an intermembrane space protein, may be imported through a unique pathway. In order to further analyze the membrane translocation step we have translated truncated mRNAs of CCP and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH), a matrix protein, in rabbit reticulocyte lysates. The resultant peptides are covalently attached to a tRNA molecule. They remain import competent upon addition of isolated mitochondria. P5CDH-tRNA was shown to be intimately associated with membrane proteins in that 6M urea was required to extract it. Similar analyses are being carried out with preCCP.

H 110 CHARACTERIZATION OF A NEW MITOCHONDRIAL IMPORT MUTANT WHICH ALSO DISPLAYS A DEFECT IN THE CELL DIVISION CYCLE, Barbara J. Smith and Michael P. Yaffe, Department of Biology, U.C. San Diego, La Jolla, CA 92093, We have previously characterized a temperature-sensitive (ts) yeast mutant that displays defects in both mitochondrial protein import and progress through the cell cycle. Genetic analysis demonstrated that the growth, import and cell cycle defects in *mas3* cells are due to a single, recessive, nuclear mutation. We have isolated a 12.6kb genomic clone which complements the ts, import and cell cycle phenotypes and maps to the *mas3* locus. Sequence analysis of a 5.8kb subclone which complements the ts mutation revealed two open reading frames (ORF's) which share 190bp of overlapping sequence corresponding to regions encoding the amino-termini of both potential proteins. ORF1 encodes a protein with a putative mitochondrial targeting signal and several membrane spanning regions, while ORF2 does not appear to contain characteristic targeting or membrane spanning regions. A replacement of the 1.1kb region containing the overlap and additional sequences with the *URA3* gene in a diploid created a recessive lethal mutation, indicating that at least one of the open reading frames encodes an essential gene. We are currently constructing various deletion and insertion mutations in order to delineate the role(s) of the ORF(s) in protein import and the cell cycle.

H 111 STUDIES ON THE ASSEMBLY OF NUCLEARLY AND MITOCHONDRIALLY ENCODED PROTEINS OF NADH DEHYDROGENASE, Robin E. Hall and James F. Hare, Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, OR 97201. The assembly of mitochondrially and cytoplasmically translated subunits of the first enzyme in the electron transport chain of the inner mitochondrial membrane, NADH dehydrogenase, was studied in rat hepatoma cultures. The rate of appearance of component proteins was measured by incorporation of pulse-labeled proteins into immunoprecipitable enzyme. Nuclear coded, imported polypeptides appear immediately after a [^{35}S]methionine pulse and retain constant stoichiometry. Mitochondrially coded proteins, although rapidly translated, peak at different times between 0 and 12 h of chase in the immunoprecipitated enzyme. Synthesis and import of nuclear coded proteins is necessary for mitochondrially coded proteins to be assembled, although the reverse is not true. Excess, unassembled mitochondrially translated subunits are degraded in an oligomycin sensitive manner. These data are consistent with a model in which a scaffold of imported proteins forms the inner core of the enzyme and mitochondrially translated proteins attach to the scaffold in a time dependent manner, acting to anchor NADH dehydrogenase into the inner membrane. It appears that an ordered, sequential assembly process may be common to inner mitochondrial membrane enzymes comprised of both nuclearly and mitochondrially translated proteins.

H 112 CELLULAR COMPARTMENTATION OF MALATE DEHYDROGENASE ISOZYMES IN YEAST, Lee McAlister-Henn, Karyl Minard, and Leslie M. Thompson, Department of Biological Chemistry, School of Medicine, University of California, Irvine, CA 92717 The structural basis for differential compartmentation and metabolic function of mitochondrial and cytosolic isozymes of malate dehydrogenase have been examined by expressing altered forms of the genes for these enzymes in *Saccharomyces cerevisiae*. Major structural differences between the yeast isozymes are observed in the amino terminal regions of these proteins. The mitochondrial enzyme is expressed as a precursor form and a seventeen residue presequence is removed from the amino terminus during mitochondrial import. We have found, however, that the mature form of mitochondrial malate dehydrogenase, expressed from the cognate gene (*MDH1*) lacking the presequence coding region, is imported as efficiently as the precursor form. Thus, the presequence is dispensable *in vivo*. The cytosolic form of yeast malate dehydrogenase possesses a twelve residue amino terminal extension relative to the mature form of the mitochondrial enzyme. To test the possibility that this extension may contribute to cytosolic localization, a gene encoding this extension in frame with the coding region for the mature form of mitochondrial malate dehydrogenase has been constructed and expressed in yeast mutants lacking either cytoplasmic or mitochondrial forms of the enzyme.

H 113 SYNTHETIC CHLOROPLAST AND MITOCHONDRIAL TRANSIT PEPTIDES. Brian Austen, Dept. of Surgery, St.George's Medical School, LONDON SW17 ORE. Theoretical analyses of mitochondrial matrix transit peptides indicate that positive charges and hydrophobic residues are critical for function. Chloroplast transit peptides are less basic, but also contain many beta-hydroxy amino acids. A synthetic peptide comprising the first 24 residues of wheat ss RUBISCO and a peptide comprising the 24-residue transit sequence of rat malate dehydrogenase adopt helical structures in detergents, trifluoroethanol and acidic phospholipids. The MDH peptide increases respiration in coupled mitochondria, and causes release of the dye calcein from PC liposomes in a way that is antogonised by cadmium and zinc. Structural predictions indicate it may form a membrane-active amphiphilic helix. Proline residues in the chlorplast peptide may distort any helix that is formed. Photoreactive derivatives of these peptides cross-link to 30kDa proteins in inner membranes of mitochondria and chloroplasts.

H 114 A COMPLEX OF GROEL AND RECOMBINANT RUBISCO SMALL SUBUNIT DISSOCIATES IN THE PRESENCE OF ATP OR ATP-γ-S, Samuel J. Landry*, Carmen W. Dessauer+, Sue G. Bartlett+ and Lila M. Gierasch*, *Dept. of Pharmacology, Univ. of Texas SW Medical Center, Dallas, TX 75235 and +Dept. of Biochemistry, Louisiana State University, Baton Rouge, LA 70808 *Escherichia coli* GroEL is a member of the chaperonin familiy of proteins, including mitochondrial HSP60 and the chloroplast subunit binding protein. Chaperonins interact with unfolded/unassembled proteins at intermediate stages of translocation across membranes or assembly into multisubunit complexes. We are investigating the interactions of GroEL with a recombinant form of a protein imported into chloroplasts, the small subunit of RuBisCO. GroEL is tightly associated with RuBisCO small subunit or its precursor expressed in *E. coli* as a fusion to *S. aureus* protein A. The complexes are retained on IgG-agarose [Landry and Bartlett *JBC* **264**, 9090 (1989)]. Other workers have shown that ATP but not the non-hydrolyzable ATP analog, AMP-PCP, affects the formation or stability of GroEL protein complexes [Gujuluva et al., *JBC* **261**, 12414 (1986) and Bochkareva et al., *Nature* **336**, 254 (1988)]. In contrast, we have found that the GroEL-RuBisCO subunit complex dissociates in the presence of the non-hydrolyzable ATP analog, ATP-γ-S, as well as ATP. Another protein of apparent MW 70,000 copurifies with the fusion protein in minor amounts and also dissociates in the presence of ATP, but less well in the presence of ATP-γ-S. Chloroplast subunit binding protein in a chloroplast extract binds to the GroEL-depleted protein A-RuBisCO subunit fusion, indicating the behavior of the GroEL-RuBisCO subunit complex may be relevant to the function of this chaperonin *in vivo*. We have initiated studies on the conformational states of GroEL to characterize GroEL-substrate interactions.

H 115 ASSEMBLY OF A NUCLEAR-ENCODED THYLAKOID MEMBRANE PROTEIN IS A MULTISTEP PROCESS INVOLVING SEVERAL REACTIONS IN THE CHLOROPLAST STROMA. Kenneth Cline and Luis A. Payan, University of Florida, Gainesville, FL. The light-harvesting chlorophyll a/b protein (LHCP) is one of several integral thylakoid membrane proteins that are synthesized in the cytosol as precursors and subsequently imported into chloroplasts. Experiments with selective inhibitors as well as time course analyses employing a rapid stopping technique reveal an assembly pathway that includes: **a. transport across the double envelope membrane and immediate processing to mature size (25 Da); b. assembly into a soluble 90 kDa complex in the organelle matrix (the stroma); and c. insertion into the thylakoid membrane.** Assembly into the 90 kDa complex as well as insertion into thylakoids can be reconstituted with chloroplast subfractions. Complex formation requires a high molecular weight stromal component, is stimulated by Mg ions, but doesn't require ATP. Thylakoid insertion requires thylakoids, energy in the form of ATP and pmf, and a stromal protein. When stroma is fractionated by gel filtration, insertion activity coelutes with 90 kDA complex forming activity. However, these activities are not identical. Isolation of the 90 kDa complex demonstrates that although complex formation maintains LHCP in an insertionally competent form, insertion into the membrane still requires stroma, indicating an additional stroma-mediated reaction. These and other observations suggest a multistep, soluble intermediate assembly pathway that involves 3 different reactions in the stroma. Comparison of the trans-envelope transport process with that of insertion into thylakoids reveals two protein translocation systems with strikingly different characteristics.

H 116 MUTANT RECOMBINANT RICIN B CHAIN UNABLE TO BIND GALACTOSE, J. Michael Lord, Peter T. Richardson, Hugh R. Woodland and Lynne M. Roberts, Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, U.K. The plant cytotoxin ricin is a heterodimer consisting of a catalytic polypeptide which inactivates 80S ribosomes (A chain) disulfide-linked to a galactose-binding lectin (B chain). The B chain has two roles in delivering the A chain into the target cell cytoplasm: it binds to cell surface galactosides and it facilitates the membrane translocation of A chain from the trans Golgi cisternae into the cytoplasm. Recently it has been proposed that the B chain translocation activity requires intracellular galactose binding, although this conclusion is controversial at present. To explore this possibility further, we have used the cloned ricin B chain gene, oligonucleotide site-directed mutagenesis and expression in a heterologous eukaryotic system to produce soluble, mutant ricin B chain no longer able to bind galactose. The ability of this mutein to facilitate translocation of ricin A chain has been assessed.

H 117 PEROXISOMAL MUTANTS OF SACCHAROMYCES CEREVISIAE, Wolf-H. Kunau, Ralf Erdmann, Jörg Höhfeld, Daphne Mertens, and Franziska Wiebel, Dept. Physiological Chemistry, Ruhr-University, D-4630 Bochum, FRG. Peroxisome proliferation by oleate[1] and a recently described screening procedure for peroxisomal mutants[2] allow to study peroxisome biogenesis in S.cerevisiae. Ten mutants defective in peroxisome assembly (pas mutants) will be described. They fall into at least six complementation groups. As a first step to dissect the mechanism of peroxisome biogenesis the PAS1 gene has been cloned by functional complementation of a pas1 mutant. The gene contains an open reading frame encoding 1043 amino acids. The predicted protein shares a region of about 150 amino acids of a remarkable degree of similarity (> 50%) with regions of the two fusion proteins Sec18p and NSF. This region which is conserved in all three proteins may represent a functional domain necessary for a fundamental step in protein sorting. By means of antibodies raised against a Pas1-ß-galactosidase fusion protein the Pas1p has been identified. A preliminary characterization will be reported.

1 Veenhuis, M., Mateblowski, M., Kunau, W.-H., and Harder, W. (1987) Yeast 3, 77-84
2 Erdmann, R., Veenhuis, M., Mertens, D., and Kunau, W.-H. (1989) Proc.Natl.Acad.Sci., USA 86, 5419-5423

H 118 TRANSLOCATION OF MICROINJECTED PROTEINS INTO THE PEROXISOMES OF MAMMALIAN CELLS. Paul A. Walton♣, Stephen J. Gould♦, James R. Feramisco♣, and Suresh Subramani♦. ♣Cancer Center and ♦Department of Biology, University of California at San Diego, La Jolla California 92093.

The aim of these studies is to gain further insight into the mechanism of protein translocation into peroxisomes *in vivo*. To this end, we have developed an assay in which peroxisomal translocation of proteins microinjected into mammalian cells can be followed by immunofluorescence microscopy. The peroxisomal protein alcohol oxidase (E.C. 1.1.3.13), a flavin-containing homo-octamer of molecular weight 630 kdal from yeast (*Pichia pastoris*) can be seen to translocate into the peroxisomes in a time-dependent manner. Indirect immunofluorescence of BALB/c 3T3 cells microinjected with this protein show a diffuse cytoplasmic staining at time zero and 20 minutes. At 40 minutes, brightly staining punctate peroxisomes appear and by 60 minutes the cytoplasm fluorescence is dimmed with prominent peroxisomal staining. Translocation of alcohol oxidase occurs using the holoenzyme or enzyme which has been thermally denatured. Control injections of non-peroxisomal proteins show no such punctate staining. This assay should be useful for addressing many questions regarding peroxisomal protein import, such as whether protein unfolding or membrane gradients are involved. In addition, these studies support the idea that the process of peroxisomal protein import has been highly conserved through evolution and that import does not involve irreversible modification of peroxisomal proteins.

H 119 PICHIA PASTORIS: A MODEL SYSTEM FOR PEROXISOME BIOGENESIS, Dannel McCollum, Stephen J. Gould, and Suresh Subramani, Dept. of Biology, B-022, UCSD, La Jolla, CA 92093. In order to better understand the processes of peroxisome biogenesis, we are pursuing a genetic analysis of this problem in the homothallic yeast *Pichia pastoris*. This yeast is particularly suitable for the study of peroxisome function as it requires peroxisomal enzymes for growth on methanol, ethanol, or oleic acid. In addition, recent results have shown that most molecular genetic techniques possible in *S. cerevisiae* are also possible in *P. pastoris*. Since growth of *P. pastoris* on either methanol, ethanol, or oleic acid each requires a different set of peroxisomal enzymes, cells that cannot grow on any of these carbon sources, but can grow on glycerol (which does not require peroxisomal enzymes) are candidiates for peroxisomal assembly (*pas*) mutants.

Mutagenized cells were initially screened for those that could grow on glycerol but not on methanol. The resulting mutants were tested for inability to grow on ethanol and oleic acid. Thus far five complementation groups have been isolated that are methanol, ethanol, and oleic acid non utilizers (classified as *meo* mutants). Biochemical characterization and immunofluorescence analysis of the meo mutants will be presented.

H 120 CAN THE SERINE-LYSINE-LEUCINE PEROXISOMAL TARGETING SIGNAL FUNCTION AT INTERNAL LOCATIONS WITHIN PROTEINS? John A. Heyman, Stephen J. Gould, and Suresh Subramani, Department of Biology, B-022, UCSD, La Jolla, CA 92093. The tripeptide serine-lysine-leucine, when present at the carboxy-terminus of a protein, has been shown to act as a peroxisomal targeting signal. Though most peroxisomal proteins contain this or a similar sequence at their C-terminus, there are many which do not. However, most of these peroxisomal proteins contain this three amino-acid sequence (or a conservative variant) at at least one internal location. We have sought to address the question of whether internal serine-lysine-leucine sequences are involved in targeting of these proteins into peroxisomes. We present data on the role of these sequences in the import of rat thiolase. In addition, we have inserted the serine-lysine-leucine sequence into several places within the polypeptide chain of mouse DHFR and describe the effects of these sequences on the subcellular distribution of this protein.

H 121 CONSERVATION OF PEROXISOMAL TARGETING SIGNAL FUNCTION AND STRUCTURE. Stephen. J. Gould, Gilbert-Andre Keller, Ben Distel, Henk Tabak, and Suresh Subramani, Dept. of Biology, B-022, UCSD, La Jolla, CA 92093. We have previously identified the minimal peroxisomal targeting signal (PTS) in luciferase as the C-terminal three amino acids of the protein, serine-lysine-leucine. We have also demonstrated that luciferase is transported to peroxisomes in mammalian, insect, plant and yeast cells. We now show that the same signal used for import into mammalian peroxisomes is used for import into yeast peroxisomes. In addition, we have raised antibodies that specifically react with the peroxisomal targeting signal. These antibodies can be used to detect peroxisomes in yeast, Neurospora, Trypanosome, plant, frog, and mammalian cells by electron microscopy and peroxisomal proteins from these organisms in Western blots. Together, these data demonstrate that the tripeptide PTS identified in luciferase can function in diverse species and that endogenous peroxisomal proteins from these organisms often contain this form of PTS.

H 122 PEROXISOME-DEFICIENT MUTANTS OF HANSENULA POLYMORPHA, James Cregg,[1] Marten Veenhuis,[2] Grietje Sulter,[2] Ida van der Klei,[2] & Wim Harder.[2] [1]Dept. of Chem. and Biol. Sci., Oregon Graduate Center, Beaverton, OR 97006, [2]Dept. of Microbiology, University of Groningen, Haren, The Netherlands. As a first step in a genetic approach toward understanding peroxisomal biogenesis and function, peroxisome-deficient mutants of the methylotrophic yeast, Hansenula polymorpha, were isolated. This organism is ideally suited for this purpose since both classical and molecular genetic techniques are available and its ability to metabolize a number of carbon and nitrogen sources is known to involve peroxisomal enzymes. Examples of such enzymes include: alcohol oxidase, dihydroxyacetone synthase and catalase (methanol utilization); malate synthase and isocitrate lyase (ethanol utilization); and amine oxidase (methylamine utilization). To look for peroxisome-deficient mutants, a collection of methanol-utilization-defective mutants was examined by electron microscopy. Two mutants were found that appear to be totally devoid of peroxisomes. Genetic analyses of the mutants indicated that they contain recessive mutations in different nuclear genes, which we have named PER1 and PER2. In both mutants, peroxisomal matrix enzymes were active but located in the cytosol. Interestingly, although the PER mutants were not capable of growth on methanol, they were capable of growth on ethanol and methylamine. These results raise the questions: Why do cells compartmentalize these pathway enzymes and, if peroxisomes are not essential for the function of some metabolic pathways, why is their use so ubiquitous?

H 123 IMPORT AND ASSEMBLY OF CANDIDA TROPICALIS HYDRATASE-DEHYDROGENASE-EPIMERASE INTO PEROXISOMES OF CANDIDA ALBICANS: R.A. Rachubinski, J.D. Aitchison, Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5 [Spon. by R.W.H. Lee]
In vivo expression of cloned genes encoding peroxisomal proteins is a valuable method to study the mechanism of peroxisomal protein targeting. Certain Candida species are useful organisms for such studies, as the formation of peroxisomes can be induced by growth on oleic acid and repressed by growth on glucose. The C. tropicalis gene encoding peroxisomal hydratase-dehydrogenase-epimerase (HDE) was inserted into the C. albicans ARS, URA 3 containing vector pMK22. C. albicans (ura 3) was transformed to uracil prototrophy. Transformants were confirmed by Southern analysis. Western blotting of yeast lysates showed the presence of HDE (100 kDa) in transformed cells. Control cells did not contain an immunoreactive polypeptide of this molecular mass. However, a weak reaction was detectable in both control and transformed cells at 97-98 kDa; presumably corresponding to endogenous HDE. Western blots also indicated increased amounts of HDE in cells grown on oleic acid as opposed to glucose. Analysis of the purified peroxisome fraction isolated from transformed C. albicans showed C. tropicalis HDE to be imported and assembled into an enzymatically active form. Experiments are currently underway using deletion mutants of HDE and DHFR-HDE fusions in an attempt to localize the peroxisomal targeting signal of HDE.

H 124 PEROXISOMAL PROTEIN IMPORT IN A METHYLOTROPHIC YEAST, Hans Hansen, Marten *Veenhuis and Rainer Roggenkamp, Institute of Microbiolgy, University of Düsseldorf, Federal Republic of Germany and *Laboratory for Electron Microscopy, Biological Centre, University of Groningen, 9751 Haren, The Netherlands. The methylotrophic yeast Hansenula polymorpha induces large peroxisomes when grown on methanol as a carbon source. Peroxisomal proteins like methanol oxidase and dihydroxyacetone synthase (DAS) do not contain a carboxy-terminal SKL sequence that was shown to serve as a target signal in other eukaryotic cells (Gould et al. (1989) J.Cell Biol. 100: 1657). However, a stretch of 12 amino acids containing SKL could be used for efficient transport of the bacterial β-lactamase (BLA) into the peroxisomes of H. polymorpha as verified by isolated peroxisomes and immuno-cytochemical staining of thin sections. Deletion of the carboxy terminus (37 amino acids) of DAS abolished import of the protein. Fusion of this amino acid stretch to BLA mediates transport of the heterologous protein into peroxisomes. Fusion of the N-terminus of DAS (115 amino acids) to BLA did not result in protein import. Therefore, the DAS protein obviously contains a carboxy-terminal target signal that will be further characterized.

H 125 BIOGENESIS OF GLYCOSOMAL MICROBODIES OF *TRYPANOSOMA BRUCEI*. Marilyn Parsons, Keith Alexander, Barbara Nielsen, and Teresa Hill, Seattle Biomedical Research Institute, 4 Nickerson St., Seattle, WA 98109.
Glycosomes are microbody organelles that compartmentalize the first seven enzymes of glycolysis in kinetoplastid protozoa. African trypanosomes, which during their life cycle are alternately parasites of mammals and tsetse flies, show stage regulation of the protein composition of glycosomes. By biosynthetic labelling and two-dimensional gel analysis we have demonstrated that this stage regulation is present at the level of protein synthesis. As a model system, we have analyzed the phosphoglycerate kinase (PGK) isozymes of *T. brucei*. Osinga et al. (Embo J. 4, 3811, 1985) demonstrated that the DNA sequence of the cytosolic and glycosomal isozymes are very similar. Both the C-terminus and two highly basic regions (hot spots) present in glycosomal PGK have been hypothesized to be important in targeting to the glycosome. Sequence analysis of the alleles expressed in our strain indicate gene conversions between the two isogenes have rendered them more similar. The hot spot 1 region is altered in the glycosomal isozyme, and the hot spot 2 sequence is also present in the cytosolic isozyme. Cloned glycosomal PGK translated in the reticulocyte lysate system is able to bind to purified glycosomes, while cytosolic PGK is not. Enzymatically active glycosomal PGK has been expressed and purified from *E. coli* and will be used to develop in vitro systems for studying glycosomal targeting.

H 126 COMPARTMENTALIZATION OF GLYCOLYSIS IN TRYPANOSOMA BRUCEI, Paul A.M. Michels, Mia Callens, Dominique Cottem, Sylvie Allert, Martine Marchand and Fred R. Opperdoes, International Institute of Cellular and Molecular Pathology, Brussels, Belgium.
In trypanosomes a unique form of metabolic compartmentalization is found: most of the enzymes of the glycolytic pathway are contained within a microbody-like organelle, the glycosome. The glycosomal enzymes are encoded in the nucleus; they are synthesized on free ribosomes in the cytosol and imported posttranslationally without any detectable form of processing. We have cloned and analyzed the genes for eight glycolytic enzymes of T.brucei: five coding for enzymes located in the glycosome and three for cytosolic enzymes. Comparison of the predicted amino-acid sequences and the sequences of the glycolytic enzymes of other organisms revealed some features specific for the glycosomal proteins. These features are: 1. Unique extensions, either at the N- or C-terminus, or unique insertions. 2. Clusters of positively charged residues, presumably present in a specific configuration on the surface of the proteins. 3. Short stretches of amino acids with significant sequence similarity, present either in an extension or internally in the polypeptides. We will present the details of these unique features, as well as the results of our current studies on their role in the translocation of the glycolytic proteins across the glycosomal membrane and the functioning of these enzymes within the organelle.

H 127 TARGETING OF PROTEINS TO THE GLYCOSOME OF *TRYPANOSOMA BRUCEI*. Christine E. Clayton, The Rockefeller University, 1230 York Ave, New York, NY 10021, USA.

Most of the glycolytic enzymes in *Trypanosoma brucei* are compartmentalized in a peroxisome-like organelle, the glycosome. Attempts to determine glycosomal targeting signals have been hampered by difficulties in developing reliable *in vitro* assays, and the complete absence of DNA transfection methodology for the organism. We have now developed methodology and vectors for expressing foreign DNA in *T. brucei* and are using the technique to determine glycosomal targeting signals. Results of these experiments will be presented.

H 128 CHARACTERIZATION OF THE *IN VITRO* IMPORT OF 3-PHOSPHO- GLYCERATE KINASE INTO THE GLYCOSOME OF *TRYPANOSOMA BRUCEI*. Jürg M. Sommer*, Julia A. Thissen*, Marylin Parsons§ and C.C Wang*, * Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94117, § Seattle Biomedical Research Institute, Seattle, WA 98109. The *in vitro* protein import assay developed by Dovey *et. al.* (1988) measures the uptake of ^{35}S-methionine-labeled *Trypanosoma brucei* glycosomal 3-phosphoglycerate kinase (gPGK) by glycosomes isolated from the long-slender bloodstream form of *T. brucei*. In further characterizing this assay we have found that up to 20% of the translated protein was in a highly aggregated form which bound preferentially to the glycosomal fraction under the assay conditions. This problem was resolved by the inclusion of 50 mM potassium acetate in the assay. Furthermore, occasional DNA contamination of the glycosome preparations tended to introduce errors in the assay. This was corrected by pretreating the glycosomes with DNase. In the modified assay system, up to 50% of the total ^{35}S-gPGK in the glycosomal fraction is resistant to either extraction by 3M urea or treatment with 500 µg/ml proteinase K. The urea resistant, glycosome-associated ^{35}S-gPGK can be chemically cross-linked to the glycosomal core proteins to form an SDS-resistant complex, suggesting that it must be in close proximity to the intraglycosomal protein matrix. However, while about half of the glycosome-associated^{35}S-gPGK can be further solubilized by either 4M urea or at pH 9.5, the intraglycosomal gPGK requires 4.5M urea or a pH of 10.5 to be released from the dense glycosomal protein core by 50%, indicating that the ^{35}S-gPGK has not been fully incorporated into the glycosomal core. The initial rate of ^{35}S-gPGK import and the amount of glycosome-associated ^{35}S-gPGK after 30 min is temperature dependent, differing by about two-fold between 4°C and 30°C. An ATP-regenerating system is not required in the assay, and the non-hydrolyzable analogs, AMP-PNP or g -S-ATP appear to have no effect , suggesting that substantial ATP hydrolysis may not be required or may only be required inside the glycosome. Complete denaturation of the newly synthesized ^{35}S-gPGK in 8M urea just prior to the assay enhances the efficiency of the apparent import. Together, these results indicate that ^{35}S-gPGK may be translocated across the glycosomal membrane in its unfolded form independent of an external energy source, and that it is probably not fully incorporated into the glycosomal protein core immediately following the import .

H 129 SIGNAL PEPTIDES BLOCK PROCESSING OF ALPHA-MATING FACTOR IN YEAST BY BINDING TO A MICROSOMAL 52kDa PROTEIN. Olwyn Westwood, Dept.of Surgery, St.George's Medical School, LONDON SW17 ORE.

Although genes related to that encoding the 54kDa subunit of SRP have been found in microorganisms, there has so far been no biochemical characterisation of signal recognition proteins in yeast. The signal region of ovalbumin, isolated as a tryptic fragment, has been found to inhibit processing and glycosylation of pre-pro alpha mating factor translated in a yeast lysate in the presence of yeast microsomes, indicating that proteins acting as receptors for signal peptides are present. A receptor protein in yeast microsomes has been identified as a 52kDa protein by cross-linking to a photoreactive signal peptide. The cross-linked protein is not extracted by 0.1M carbonate, but is solubilised by octyl glucoside, and thus behaves like an integral membrane protein. Cross-linking is enhanced in microsomes isolated from a sec 61 mutant strain provided by Dr.R.Scheckman at UC Berkeley.

H 130 SACCHAROMYCES CEREVISIAE AND SCHIZOSACCHAROMYCES POMBE CONTAIN A HOMOLOGUE TO THE 54 KD SUBUNIT OF THE SIGNAL RECOGNITION PARTICLE THAT IN S. CEREVISIAE IS ESSENTIAL FOR GROWTH. Byron C. Hann, Mark A. Poritz and Peter Walter, Department of Biochemistry and Biophysics University of California, Medical School San Francisco, CA 94143-0448.

We have isolated and sequenced genes from S. cerevisiae ($SRP54^{sc}$) and S. pombe ($SRP54^{sp}$) encoding proteins homologous to both the 54 kd protein subunit ($SRP54^{mam}$) of the mammalian signal recognition particle (SRP) and the product of a gene of unknown function in E. coli, ffh (Römisch et al., Nature 340, 478-482 (1989); Bernstein et al., Nature 340, 482-486 (1989)). To accomplish this we took advantage of short stretches of conserved sequence between ffh and $SRP54^{mam}$ and used the polymerase chain reaction (PCR) to amplify fragments of the homologous yeast genes. The DNA sequences predict proteins for $SRP54^{sc}$ and $SRP54^{sp}$ which are 47% and 52% identical to $SRP54^{mam}$, respectively. Like $SRP54^{mam}$ and ffh, both predicted yeast proteins contain a GTP binding consensus sequence in their N-terminal half (G-domain), and Met-rich sequences in their C-terminal half (M-domain). In contrast to $SRP54^{mam}$ and ffh the yeast proteins contain additional Met-rich sequences inserted at the C-terminal portion of the M-domain. $SRP54^{sp}$ contains a 480 nucleotide intron located 78 nucleotides from the 5' end of the open reading frame. Although the function of the yeast homologues is unknown, gene disruption experiments in S. cerevisiae show that the gene is essential for growth. The identification of $SRP54^{sc}$ and $SRP54^{sp}$ provides the first evidence for SRP related proteins in yeast.

H 131 OLIGOSACCHARIDE PROCESSING IN THE FISSION YEAST, SCHIZOSACCHAROMYCES POMBE, T. G. Chappell, L. J. Page and G. Warren, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX United Kingdom.

A membrane associated galactosyltransferase has been purified to homogeneity from the fission yeast, *S. pombe*. The enzyme has a molecular weight of 61,000 and is capable of transferring galactose from UDP–galactose to a variety of mannose–based acceptors to form an α–1,2 galactosyl mannoside linkage. Confocal immunofluorescence localization of the purified galactosyltransferase with monospecific antibodies shows the enzyme to be present in discrete organelles within the cytoplasm of fission yeast. The organelles are consistent in size and number with the stacked, cisternal Golgi seen in electron micrographs.

We have also characterized a mannosyltransferase activity present in fission yeast microsomes. The two transferase activities can be separated by sucrose density centrifugation, and both are found in membranes with densities similar to those found for mammalian Golgi. We are currently in the process of purifying the mannosyltransferase activity.

In an attempt to study the signals involved in localizing transferases within the fission yeast Golgi, we have isolated the gene for galactosyltransferase. We are currently doing mutational analysis to analyze the targetting of the gene product to the Golgi.

H 132 IDENTIFICATION OF ESSENTIAL DOMAINS WITHIN A PHO5-LacZ HYBRID PROTEIN REQUIRED TO BLOCK PROTEIN TRANSLOCATION ACROSS THE ENDOPLASMIC RETICULUM IN YEAST. Paul B. Wolfe and Matthew R. Young, Department of Biological Chemistry, University of Maryland at Baltimore, Baltimore, MD 21201.

We have constructed a set of hybrid proteins composed of amino-terminal residues of the yeast repressible acid phosphatase (PHO5) and bacterial β-galactosidase (lacZ). When expressed in yeast, these PHO5-LacZ fusion proteins block secretion of several secretory pathway precursors, including acid phosphatase, the mating pheromone α-factor, and the vacuolar protease carboxypeptidase Y. The block occurs after targeting but before translocation across the endoplasmic reticulum. Evidence suggests that the PHO5-LacZ fusion proteins block translocation by competing with secretory pathway precursors for limiting amounts of a factor required for efficient translocation.

To define the structural elements of the fusion protein responsible for mediating the translocation block, we have constructed a series of deletion derivatives of the largest PHO5-LacZ hybrid. Deletion analysis has identified two amino acid sequences in the PHO5 coding region of the hybrid which are required to confer the block in translocation. Secondary structure analysis of these regions shows that each contains a sequence with a high potential to form a β-turn. To test the role of this type of secondary structure in blocking protein translocation, we plan to alter the β-turn potential of these domains by site-directed mutagenesis. Mutations which reduce the β-turn potential and also eliminate the hybrid protein phenotype would suggest that this secondary structure is required to confer the hybrid-dependent block in translocation. If so, the limiting factor may be required to prevent premature formation of β-turns in nascent secretory precursors.

H 133 ISOLATION AND CHARACTERISATION OF YEAST ENDOPLASMIC RETICULUM.

Christopher M. Sanderson, Joanne S. Crowe, David I. Meyer, Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024. We have created a physical marker for the rough endoplasmic reticulum (RER) in the yeast *S. cerevisiae*. Human ribophorin I, a RER specific protein when expressed in *S. cerevisiae*, is targeted to and concentrated within translocation-competent membranes. Using this marker together with two other putative yeast ER proteins, BiP and SEC 62, we have isolated rough and smooth membranes which are physically and functionally distinct. In order to identify proteins involved in translocation we have further purified yeast RER derived membranes. As in mammalian cells, chemical removal of ribosomes results in a unique density perturbation that specifically shifts RER derived membranes away from contaminating membranes of similar buoyant density. Membranes isolated by this method are translocation competent and contain a set of unique proteins that may well play a role in translocation.

H 134 *SEC63*, A YEAST GENE WITH HOMOLOGY TO THE *E. COLI* DNA J HEAT SHOCK PROTEIN, IS REQUIRED FOR PROPER INSERTION OF SECRETORY PROTEINS INTO THE ENDOPLASMIC RETICULUM, Jonathan Rothblatt*, David Feldheim, and Randy Schekman, Division of Biochemistry & Molecular Biology, University of California, Berkeley, CA 94720 and *Dept. of Biological Sciences, Dartmouth College, Hanover, NH 03755

Genes that function in translocation of secretory protein precursors into the ER have been identified by a genetic selection for mutant yeast cells that fail to translocate a signal peptide-cytosolic enzyme hybrid protein. The mutant, *sec63*, is thermosensitive for growth and accumulates a variety of secretory and vacuolar precursors that lack post-translational modifications indicative of insertion into the ER. Some form of interaction among the *SEC61*, *SEC62* and *SEC63* gene products is suggested by the observation that haploid cells containing any pair of the mutations are inviable at 24°C and show a marked enhancement of the translocation defect. The translocation defect of the *sec63* mutant has been reproduced *in vitro*. Microsomes from *sec63* cells display low and thermolabile translocation activity for prepro-α-factor synthesized with a cytosol fraction from wild-type yeast. Cloning and DNA sequencing of *SEC63* (Sadler *et al.* 1989. *J. Cell Biol.* **109**, in press) has revealed a 76 amino acid domain that is homologous to a region of *E. coli* DnaJ protein, a heat shock protein that works in concert with the 70kD heat shock protein, DnaK, to catalyze bacteriophage lambda replication. We have constructed a collection of Sec63p-invertase gene fusions to examine the topology of Sec63p in the ER membrane. Preliminary analysis indicates that the DnaJ homology region faces the lumen of the ER. Sec63p may interact with the *KAR2* gene product, an ER lumenal protein homologous to the mammalian 70kD heat shock protein BiP, that appears to be required also for translocation of proteins into the yeast ER (Rose *et al.* 1989. *Cell* **57**: 1211-1221).

H 135 SEARCH FOR CONDITIONAL MUTATIONS IN THE 7SL RNA OF THE SIGNAL RECOGNITION PARTICLE (SRP) FROM *YARROWIA LIPOLYTICA*, David M. Ogrydziak, Debbie S. Yaver and Sam Matoba, Institute of Marine Resources, University of California, Davis, CA 95616

The yeast*Y. lipolytica* has two functional genes (*SCR1* and *SCR2*) which code for the putative SRP 7SL RNAs. At least one functional 7SL RNA is required for growth. In order to confirm that *SCR1* codes for a component of SRP and to provide *in vivo* evidence that SRP has a role in protein secretion, conditional lethal mutations in *SCR1* will be isolated and their effects on processing and secretion of alkaline extracellular protease will be examined. Two *SCR1* genes with mutations in a highly conserved stem loop region (Campos et al., NARS, *17*, 1573, 1989) which most likely interacts with the 19 K polypeptide of SRP have been constructed by site-directed *in vitro* mutagenesis. The mutations (*SCR1-1*, G->A at position 129 and A->T at position 131 and *SCR1-2*, deletion of nucleotides 126->135) will be introduced on a plasmid containing a *LEU2* selectable marker into a strain containing chromosomal deletions of *SCR1* and *SCR2* and wild type *SCR1* on a plasmid with a *URA3* selectable marker. Leu^+ Ura^- strains which contain only the mutated *SCR1* gene will be selected using 5-fluoroorotic acid and screened for heat and cold sensitivity and possibly for hypersensitivity to D_2O. Random hydroxylamine *in vitro* mutagenesis and the plasmid shuffle will also be used to examine a broader range of mutations.

H 136 MEMBRANE INSERTION OF THE YEAST URACIL PERMEASE, A POLYTOPIC PLASMA MEMBRANE PROTEIN. Rosine Tsapis[1], Sandra Silve[1], Catherine Garnier[1], Christiane Volland[1], Richard Jund[2] and Marie-Renée Chevallier[2]
1. Institut J. Monod, Université Paris VII, 2 Place Jussieu, 75005 PARIS, France.
2. IBMC, 15 rue R. Descartes, 67084 STRASBOURG, France.
Uracil permease, encoded by FUR4 gene, is a multispanning protein of the yeast plasma membrane. It has a long N-terminal hydrophilic segment, followed by 10 to 12 putative transmembrane segments, and an hydrophilic C-terminus. The overexpression of active uracil permease allowed us to detect the protein in cell free extracts, with the use of an antibody raised against a synthetic peptide corresponding to the C-terminal sequence. The protein does not seem to undergo important post-translational modifications, such as proteolytic processing or glycosylation, as judged by the absence of any matured forms in a pulse chase experiment, or by the absence of modification after endoglucosidase H treatment. By fusing increasing N-terminal segments of the uracil permease with signal peptide deleted acid phosphatase, we showed that the first putative transmembrane segment of the uracil permease indeed corresponds to the first signal anchor sequence. Several experimental arguments among which the absence of glycosylation of the three potential sites localized in the N-terminus of the permease allowed us to propose a cytosolic orientation of this N-terminal segment. The effect of *sec61* and *sec62* mutations on the translocation of uracil permease, or hybrid permease-phosphatase proteins, will be presented. We already know that *sec62* mutation completely prevents membrane insertion of the hybrid protein carrying the first permease transmembrane segment.

H 137 **IDENTIFICATION OF MUTANTS THAT SUPPRESS THE PHO5-LacZ HYBRID-DEPENDENT ACCUMULATION OF SECRETORY PATHWAY PRECURSORS IN YEAST.** Joanne Andreadis-Crawford and Paul B. Wolfe, Department of Biological Chemistry, University of Maryland at Baltimore, Baltimore, MD 21201.

PHO5-LacZ hybrid proteins are composed of amino-terminal residues derived from the yeast repressible acid phosphatase (PHO5) and bacterial β-galactosidase (lacZ). When expressed in yeast, these PHO5-LacZ fusions cause the accumulation of secretory pathway precursors on the cytoplasmic surface of the endoplasmic reticulum. We have developed a genetic screen to identify mutations which suppress this phenotype. Yeast strains which carry an ade2 lesion accumulate a red pigment derived from the purine biosynthetic pathway. Transformants of an ade2 strain which carry a PHO5::lacZ gene are red when grown under conditions which repress expression of the fusion protein (high phosphate medium); but remain white when grown under inducing conditions (low phosphate medium). This change in colony color is dependent on the expression of the PHO5-LacZ fusion protein and has been used to enrich for suppressors of the hybrid-dependent block in secretion.

An ade2 strain bearing an interfering PHO5::lacZ plasmid was mutagenized with EMS and grown on low phosphate medium containing limiting adenine. Red colonies were selected and analyzed for suppression of the hybrid protein phenotype by measuring β-galactosidase activity and secreted acid phosphatase activity. Forty-four candidates were identified which displayed at least 50% of hybrid protein β-galactosidase activity and restore secretion of acid phosphatase by at least 50%. Extragenic suppressors were confirmed by replacing the resident PHO5::lacZ plasmid with new plasmid. PHO5-LacZ suppressor activity was confirmed by measuring the reduction in accumulated secretory precursors by quantitative densitometry of immunoblots. These mutants will be used to identify genes which are involved in protein translocation in yeast.

H 138 **A RATE-LIMITING STEP IN THE TRANSLOCATION OF A ROTAVIRUS GLYCOPROTEIN VP7 IS MEDIATED BY ITS SIGNAL PEPTIDE PLUS DOWNSTREAM SEQUENCES,** **Gerald W. Both, S. Clare Stirzaker, and Didier Poncet,** CSIRO Division of Biotechnology, Laboratory for Molecular Biology, PO Box 184, North Ryde, NSW 213, Australia

The H2 signal sequence of the simian rotavirus glycoprotein VP7 directs the protein into the ER and has a role in retaining it there as a resident membrane protein. We have shown using *in vivo* pulse-chase experiments that VP7 glycosylation is not cotranslational. Non-glycosylated VP7 is still detectable in the ER after a 25 min chase. After pulse-labeling, both glycosylated and non-glycosylated forms of H2VP7 were recovered in microsomes but the latter was sensitive to digestion with trypsin. Thus, the nascent protein associated with the membrane but most of it was translocated into the ER post-translationally. Since the single glycosylation site in VP7 lies only eighteen residues beyond the signal peptide cleavage site, a rate-limiting step must occur very early in translocation of this protein. Bovine rotavirus VP7 which is also retained in the ER was glycosylated and translocated rapidly. Thus, the delay in translocation seen with the simian VP7 is not essential for retention of the protein in the ER. By constructing hybrids between the simian and bovine VP7 proteins we have shown that the presence of the H2 signal peptide together with residues 63-111 is required to produce delayed translocation of the simian protein. We propose that despite rapid cleavage of the signal peptide, these sequences interact to modulate the translocation of the protein. The data are consistent with the idea that certain proteins may be inserted into the ER in a loop configuration (Shaw, A.S., P.J.M. Rottier and J.K. Rose. 1988. Evidence for the loop model of signal sequence insertion into the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* 85: 7592-7596).

H 139 SECRETION OF PROTEINS FROM PLANT CELLS

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Secretion of proteins from cells occurs by recognition of an amino terminal peptide extension that ultimately results in the translation of the targeted protein directly through the endoplasmic reticulum (ER) membrane and into the ER lumen. From the ER lumen the protein can either be retained in the lumen or processed further through the Golgi and eventually be secreted or targeted to the vacuole. We are currently examining the parameters that are important in endomembrane targeting of proteins in plant cells. A series of gene fusions have been constructed to study both the requirements of the signal peptide and of the protein being targeted. The proteins being targeted include beta-glucuronidase (GUS), neomycin phophotransferase (NPTII), and chloramphenicol acetyl transferase (CAT). We have used site directed mutagenesis to examine the role of glycosylation in the targeting of GUS (two potential glycosylation sites) and CAT (one potential glycosylation site). We are also examining the efficacy of various bacterial, animal and plant signal peptides.

H 140 THE CANINE SIGNAL PEPTIDASE COMPLEX CONTAINS TWO HOMOLOGOUS YEAST SEC11-LIKE SUBUNITS, Gregory S. Shelness* and Günter Blobel, Laboratory of Cell Biology, Rockefeller University, New York, NY 10021. *Current address: Molecular Genetics Research Program, Dept. of Comparative Medicine, Wake Forest University Medical Center, Winston-Salem. NC 27103

Canine microsomal signal peptidase activity was previously found to purify as a complex of five proteins with molecular masses of 25, 22/23, 21, 18 and 12 kDa. Two of the signal peptidase complex (SPC) proteins have been cloned and sequenced. The differentially glycosylated subunit (22/23 kDa) was found to have no sequence similarity to other known proteins. However, the 21 kDa protein was observed to be a mammalian homolog of SEC11, a protein essential for signal peptide processing and cell growth in yeast. We have now cloned and sequenced the 18 kDa subunit of the SPC and find that it is also a SEC11 homolog. Both the 18 and 21 kDa SEC11 homologs are integral membrane proteins by virtue of their resistance to alkali extraction. Upon detergent solubilization of salt and EDTA extracted rough microsomes (RMs), both proteins remain quantitatively complexed with the 22/23 kDa glycoprotein subunit. However, carbonate extraction of RMs prior to solubilization, or mild urea treatment, causes partial dissociation of the the SPC. The SEC11-like proteins displaced from the complex under these conditions demonstrate no signal peptide processing activity. The existence of homologous subunits is common to a number of known protein complexes and provides further evidence that the association between SPC proteins observed in vitro may be physiologically relevant to the mechanism of signal peptide processing and perhaps protein translocation.

H 141 STRUCTURAL FEATURES IN THE NH_2-TERMINAL REGION OF THE HUMAN PRE(ΔPRO)APO-AII SIGNAL PEPTIDE INFLUENCE THE FIDELITY OF SIGNAL PEPTIDASE CLEAVAGE, S. F. Nothwehr and J. I. Gordon, Washington University, St. Louis, MO 63110. The structural characteristics of signal peptides which determine the specificity of co-translational cleavage by eukaryotic signal peptidase have not been fully defined. The 20 amino acid signal peptide of pre(Δpro)apo-AII contains the tripartite domain structure typical of eukaryotic prepeptides as well as multiple potential sites for co-translational processing. We previously analyzed 40 mutant derivatives of this model preprotein by using an in vitro translation/canine microsome processing assay and radiochemical sequencing of the processed products. We showed that the site of cleavage chosen by signal peptidase is influenced by (i) the physical-chemical properties of the -1 residue (small, neutral amino acids are favored) and (ii) the distance distance between the potential -1 residue and the h/c boundary (4-5 residues appear optimal). To investigate whether structural features in the NH_2-terminal region of signal peptides play a role in cleavage specificity, we have carried out mutagenesis of a pre(Δpro)apo-AII mutant, which has roughly equal cleavage between the $Gly^{18}\downarrow$ and $Gly^{20}\downarrow$ sites. By inserting various amino acids between the (+) charged n-region (NH_2-Met-Lys) and the h-region (hydrophobic core) we found that movement of the n/h boundary toward the NH_2-terminus results in dramatic shift in cleavage to the upstream site. A significant but less dramatic shift was also observed when the NH_2-Met-Lys dipeptide was moved upstream, while holding the position of the n/h boundary constant, relative to the original sites of cleavage. Replacement of the Lys^2 residue with hydrophilic/(-) charged residues resulted in preservation of the original sites of cleavage. Its replacement with a hydrophobic residue again caused cleavage to shift upstream. None of these mutations markedly altered the efficiency of translocation or cleavage. We conclude that the position and presence of the hydrophilic/charged domain at the NH_2-terminus of signal peptides affects the site of cleavage chosen by signal peptidase.

H 142 FUNCTIONAL LIMITS OF CONFORMATION AND HYDROPHOBICITY IN BACTERIAL SIGNAL PEPTIDE CLEAVAGE REGIONS, Genevieve A. Laforet* and Debra A. Kendall**
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**Department of Molecular and Cell Biology, The University of Connecticut, Storrs, CT 06269

Prokaryotic proteins destined for transport out of the cytoplasm typically contain an amino-terminal extension called the leader or signal peptide that is required for export. Signal peptides vary widely in sequence but can be divided into three characteristic regions: a positively-charged amino-terminus, a central hydrophobic core, and a carboxy-terminal cleavage region or c-region. Because of their lack of sequence homology, signal peptides are thought to mediate transport on the basis of comparatively general structural and physical properties such as charge, hydrophobicity, and conformation rather than strict sequence specificity. However, one portion of the signal peptide, the cleavage region, seems to require a higher degree of amino acid sequence stringency than the rest of the signal peptide. In particular, this segment is enriched for polar residues and the amino acids found at positions -1 and -3 preceding the cleavage site are limited to small neutral side chains. In addition to these specific sequence requirements, secondary structural characteristics have also been implicated in the function of this region. Conformational predictive schemes and mutational studies have implicated the importance of a β–turn for signal peptidase recognition and cleavage; several models of prokaryotic protein transport invoke at least the transient formation of a turn or loop structure during signal peptide insertion and/or cleavage. In this study, we have utilized evidence from physical studies in tandem with predictive schemes in an attempt to vary systematically the physical and structural parameters likely to contribute to the function of the cleavage region. Specifically, we have assessed the importance of hydrophobicity, presence or absence of a proline residue, and expected conformational preference. Using cassette mutagenesis, we have constructed polymeric, idealized sequences of varying hydrophobicities along with sequences corresponding to known secondary structures determined by physical data, all within the framework of either a wild type or idealized signal peptide. Based on this analysis, we have found that that either a potential alpha helical or beta turn structure in the cleavage region can function equally well, irrespective of the presence of a proline residue. A relatively broad range of hydrophobicities is permitted, but violation of the -1, -3 rule effectively abolishes processing and transport.

H 143 SIGNAL PEPTIDES CONTAINING POLYMERIC SEQUENCES REVEAL THE HYDROPHOBICITY REQUIREMENTS GOVERNING SPECIFIC STEPS IN PROTEIN TRANSPORT, Margaret M. Chou* and Debra A. Kendall§, *Rockefeller University and § Dept. of Cellular and Molecular Biology, University of Connecticut.

We have examined the hydrophobicity component of signal peptide function using polymeric sequences in combination with cassette mutagenesis. Using homopolymeric units of either isoleucine,leucine, valine or alanine to replace the natural core segment of the E. coli alkaline phosphatase signal peptide, the hydrophobicity requirements for export and processing were delineated. The transport properties of these mutants demonstrated that the net hydrophobicity determines the total extent of precursor processing, while a high mean hydrophobicity per residue is critical for complete, rapid processing and translocation. Moreover, alkaline phosphatase was converted from a periplasmic to an active membrane-anchored protein via a signal containing 20 leucine residues. This application of polymeric sequences allows systematic comparisons to be made, unambiguously revealing the hydrophobicity requirements governing specific steps in the transport process.

H 144 ALBUMIN REDHILL (-1 Arg, 320 Ala→Thr). A NEW GLYCOPROTEIN VARIANT OF HUMAN ALBUMIN WHOSE PRECURSOR HAS AN ABERRANT SIGNAL PEPTIDASE CLEAVAGE SITE, Stephen O. Brennan, Timothy Myles, Robert J. Peach, David Donaldson* and Peter M. George, Molecular Pathology Laboratory, Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand and *Department of Pathology, New East Surrey Hospital, Redhill, Surrey, U.K.

Albumin Redhill is an electrophoretically slow genetic variant of human serum albumin that does not bind $^{63}Ni^{2+}$ and has a molecular weight 2.5 kDa higher than normal serum albumin. Its inability to bind Ni was explained by the finding of an additional residue of Arg at position -1. This alone, however, did not explain the molecular basis of the genetic variation (since proalbumin contains paired Arg residues at -1 and -2) nor the increase in apparent Mr. We show that this observation resulted from a mutation of 320 Ala→Thr which introduces an Asn-Tyr-Thr oligosaccharide attachment sequence centered on Asn 318.

This finding still did not satisfactorily explain the presence of the additional arginine residue at position -1. DNA sequencing of polymerase chain reaction amplified genomic DNA encoding the preprosequence of albumin allowed us to identify an additional mutation of -2 Arg→Cys. This introduces a new preprosequence of M K W V T F I S L L F L F S S A Y S R G V F C R - (cf. - - Y S R G V F R R - in normal human preproalbumin). We propose that the new F C R sequence in the propeptide is an aberrant signal peptidase cleavage site and that the signal peptidase cleaves the propeptide of albumin Redhill in the lumen of the endoplasmic reticulum before it reaches the Golgi vesicles, the site of the diarginyl specific proalbumin convertase.

H 145 FUNCTION OF A HOMOLOG OF SRP54 IN E. COLI, Harris D. Bernstein and Peter Walter, Department of Biochemistry and Biophysics, University of California San Francisco, San Francisco, CA 94143-0448.
Cross-linking experiments have shown that the 54kd subunit of the signal recognition particle (SRP) recognizes signal sequences of nascent secretory and transmembrane proteins as they emerge from the ribosome during translation (Krieg, et al., 1986, PNAS 83: 8604-8608; Kurzchalia, et al., 1986, Nature 320: 634-636). Recently the gene that encodes this protein in mammalian cells has been cloned (Bernstein, et al.,1989, Nature 340: 482-486; Romisch, et al.,1989, Nature 340: 478-482). This work revealed that a long open-reading frame previously identified in E. coli (Bystrom, et al., 1983, EMBO J., 2: 899-905) is predicted to encode a protein that is homologous to SRP54 throughout its entire length. We have named the E. coli protein ffh ("fifty-four homolog"). An antibody that has been raised against a peptide of ffh specifically recognizes an E. coli protein of approximately 50kd. Progress on experiments designed to determine the intracellular localization and function of this protein will be reported.

H 146 INVOLVEMENT OF LPS IN THE ELABORATION OF PORIN PROTEINS IN ESCHERICHIA COLI K12, Joe A. Fralick, Department of Microbiology, Texas Tech University School of Medicine. Health Sciences Center, Lubbock Texas, 079430. Lipopolysaccharide (LPS) is found exclusively in the outer leaflet of the outer membrane of Gram negative bacteria and has been implicated in the assembly of this membrane through its interactions with outer membrane proteins. For instance, LPS has been implicated in the folding and translocation of pre-OmpA from the periplasmic region to the outer membrane and for the assembly of OmpF in E. coli B cells. Furthermore the levels of major outer membrane proteins have been shown to be drastically decreased in S. typhurium and E. coli K12 mutants synthesizing defective LPS molecules. In E. coli the decrease was mainly due to the almost total loss of the OmpF porin. The mechanism(s) by which OmpF or other outer memabrane proteins are affected in LPS mutants is unknown. In this study we have begun genetically analyzing the effect of mutations which alter LPS core structure on the elaboration of the related porin proteins of E. coli K12 strains; OmpC, OmpF and Lc.

H 147 EFFICIENT *IN VITRO* TRANSLOCATION AND ASSEMBLY OF PORIN INTO *E.COLI* MEMBRANES, Keya Sen and Hiroshi Nikaido, Department of Molecular and Cell Biology, U.C. Berkeley, Berkeley, CA 94720
The partitioning of proteins into the outer membrane of *E.coli* from their sites of synthesis in the cytoplasm is still unclear. Using the assembly of OmpF porin into the outer membrane, where it exists as a trimer, as a model system, we have shown that the proteins are first secreted into the periplasm before being incorporated into the outer membrane. Thus when spheroplasts from *E.coli* B/r were pulse labeled with [^{35}S] methionine, 30-80% of the newly made OmpF porin was found to be secreted into the medium. The secreted porin reacted with antimonomer OmpF monoclonal antibodies, were sensitive to trypsin and migrated as monomers when applied without heat dissociation to SDS-PAGE, indicating that they are monomeric and are in a different conformation than the trimeric form found in the outer membrane. When this secreted porin was incubated with an envelope fraction from *E.coli* or with purified lipopolysaccharides (LPS) in the presence of 0.03% Triton X-100, 65-75% of the monomeric protein was converted into the mature trimeric form. Thus these results appear to reproduce a part of the physiological export and targeting steps of the protein into the outer membrane and indicates that the interaction of porin with LPS is important in its insertion into, or its maturation, within the membrane.

H 148 ALKALINE PHOSPHATASE FUSIONS FOR STUDYING PROTEIN SECRETION IN BACILLUS, Mark Payne and Ethel Jackson, Central Research and Development Dept., E. I. du Pont de Nemours and Co., Wilmington, DE 19880-0228

The efficient secretion of certain proteins by *Bacillus* species has been commercially important for enzyme production, but the mechanism has not been studied in detail. *E. coli* alkaline phosphatase (AP) has been valuable for studying secretion in *E. coli* since AP is active only when secreted and its activity is insensitive to N-terminal fusions. In order to use AP to analyze secretion in *B. subtilis*, a vector was constructed containing the AP mature sequence fused to the expression elements and signal sequence of *B. amyloliquiefaciens* alkaline protease. *B. subtilis* harboring this vector efficiently secretes AP into the culture supernatant, and export depends on a functional signal sequence. The kinetics of this secretion were studied in detail.

This vector is used to assay the ability of *B. subtilis* to secrete various polypeptides. Heterologous protein coding sequences are inserted between the signal sequence and the AP sequence so that secretion of AP is a measure of the secretion compatibility of the N-terminal polypeptide. Such protein fusions allow use of *E. coli* AP as a secretion reporter for a variety of applications in *B. subtilis*.

H 149 TARGETING OF PLANT GLYCERALDEHYDE-3phosphatic DEHYDROGENASE TO THE OUTER MEMBRANE OF TRANSFORMED BACTERIA, Jan A. Miernyk, Seed Biosynthesis Research Unit, USDA, ARS, Northern Regional Research Center, Peoria, IL 61604

A plasmid was constructed that contains DNA encoding the signal sequence from E. coli ompA fused to the 5'-end of a cDNA for the cytoplasmic isozyme of GAPDH from castor oil seeds. This plasmid was used to study the synthesis and processing of the hybrid secretory protein in E. coli SCS1. Upon induction with IPTG the heterologous protein accumulated to more than 12% of total protein. Quantitative cell fractionation revealed that the processed, mature GAPDH was not located in the periplasm but rather associated with the outer bacterial membrane. Washing the membranes with alkaline sodium carbonate released less than 5% of GAPDH. Subsequently it was shown that GAPDH was specifically labeled when palmitate was added immediately after induction, and that processing of the fusion precursor could be inhibited by preincubation with globomycin. It appears that the addition of the signal sequence has exposed a cryptic procaryotic lipid modification site within plant GAPDH, and that the stable membrane association is the result of lipid modification rather than the occurrence of a hydrophobic membrane spanning sequence.

H 150 SECRETION OF ACTIVE KRINGLE-2-SERINE PROTEASE (K_2-SP) IN ESCHERICHIA COLI, Judith A. Diaz-Collier, Mark G. Obukowicz, Mark G. Gustafson, Kurt D. Junger, Richard M. Leimgruber, Arthur J. Wittwer, Tze-Chein Wun, Thomas G. Warren, Bruce F. Bishop, Karl J. Mathis, David T. McPherson, Ned R. Siegel, Michael G. Jennings, Blanche B. Brightwell, Les D. Bell, Charles S. Craik*, and William C. Tacon, Corporate Research and Development, Monsanto Co., St. Louis, MO 63198 and *Department of Pharmaceutical Chemistry, University of California-San Francisco, San Francisco, CA 92121

Active human tissue plasminogen activator variant kringle-2-serine protease (K_2-SP domains, referred to as MB1004) was synthesized as a secreted protein in Escherichia coli, isolated, and characterized. MB1004 is a relatively large and complex protein; approximately 38 kDa in size and containing nine disulfide bonds. Active, soluble MB1004 was secreted into the periplasm of E. coli by fusing the protein to the PhoA leader peptide expressed from either the tac or phoA promoter. Active, properly-folded MB1004 was isolated from E. coli with a recovery of ~20µg/L of broth. The N-terminus was sequenced and found to be identical to the predicted amino acid sequence of MB1004. The speific activity of purified MB1004 from E. coli was similar to that of deglycosylated recombinant material purified from human cell culture in three different assays. These data suggest that soluble, fully active MB1004 secreted by E. coli is very similar to the enzyme derived from mammalian cells.

H 151 PROTEIN PHOSPHORYLATION EVENTS IN BACTERIAL PROTEIN EXPORT, John A. Corbett and Bruce R. Copeland, Dept. of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300.

Bacterial protein export has a strict requirement for ATP. This has led to speculation that one or more components of the export machinery might be phosphorylated during export. We have recently detected in vitro phosphorylation of two proteins 69,000 and 49,000 apparent molecular weight under conditions analogous to those used for standard in vitro bacterial protein translocation assays. Phosphorylation of both proteins is dependent on inverted E. coli membrane vesicles as well as on export precursors. We have been able to elicit phosphorylation using either purified leucine-specific binding protein precursor (preLivK) or in vitro synthesized preLivK and pre-β-lactamase. Further evidence connecting phosphorylation to export comes from studies with uncouplers of membrane gradient energy and with the phosphohydrolase inhibitor vanadate, both of which inhibit translocation and phosphorylation. The stability characteristics of the phosphorylation are indicative of an acyl phosphate linkage, as evidenced by sensitivity to hydroxylamine and high temperatures. The phosphorylation is also labile under standard SDS PAGE conditions. Attempts are under way to permanently label the phosphoproteins by reductive tritiation, thereby enabling identification of the proteins and characterization of the phosphorylation sites. Preliminary data appears to indicate that the 69,000 molecular weight phosphoprotein is a chaperone for preLivK protein during export.

H 152 A MUTANT OF M13 PROCOAT PROTEIN THAT INSERTS INTO THE E. COLI MEMBRANE WITHOUT A SIGNAL SEQUENCE,
Andreas Kuhn, Department of Microbiology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland.

The M13 coat protein its synthesized as a precursor, called procoat, with a 23 amino acid long signal sequence. We have previously shown by a collection of various mutants that the procoat protein inserts into the membrane as a loop structure[1] and requires both[2], the signal sequence and the mature region as well, for its insertion.

As expected, the M13 coat protein was found in the cytoplasm when the signal sequence was deleted. When, however, the charged amino acids at positions +2, +4 and +5 are changed to neutral residues, M13 coat protein was membrane inserted. Thus, the M13 coat protein can insert by another mechanism without the formation of a transmembrane loop structure. It also shows that the M13 coat protein requires its signal sequence to translocate the charged amino acids across the membrane. Possibly, the water shell surrounding the charged residues make a signal sequence indispensible.

(1) Kuhn, A. (1987) Science 238 1413.
(2) Kuhn, A., Wickner, W. & Kreil, G. (1986) Nature 322, 335.

H 153 TWO COMPONENTS OF THE ESCHERICHIA COLI EXPORT MACHINERY, PrlA (SecY) AND PrlG (SecE), ARE STABLY ASSOCIATED DURING PROTEIN TRANSLOCATION ACROSS THE INNER MEMBRANE. K. Bieker and T. Silhavy. Department of Biology, Princeton University, Princeton, N.J., 08544.

In E. coli lacZ fusions to genes that specify exported proteins are lethal when overexpressed owing to a fatal "jamming" of the cellular export machinery. This lethality is relieved by signal sequence mutations that render the LacZ hybrid protein export-defective. Previously we have shown that the cellular component sequestered by hybrid jamming is PrlA (SecY) using an approach termed suppressor-directed inactivation (SDI). In essence, this approach exploits a suppressor of signal sequence mutations prlA4, to target an export-defective hybrid protein and specifically inactivate the suppressor protein (PrlA4) in (prlA4/prlA$^+$) heterogenotes. Because the hybrid protein jams in transmembrane orientation, we conclude that PrlA is a component of the translocator. By introducing a mutant allele that renders SecE rate-limiting for export we can use SDI to show that both PrlA and SecE are components of the translocator complex. This inference is further supported by the allele-specific, haploid lethal phenotype exhibited by certain prlA prlG (secE) strains, a condition commonly termed synthetic lethality. We conclude from these results that these two proteins PrlA (SecY) and PrlG (SecE) interact directly during the translocation reaction. We show further that SDI can be extended to examine PrlG (SecE) using the suppressor allele, prlG1. In this case, the export-defective hybrid protein ties up the suppressor protein (PrlG1) at a step in export prior to PrlA. Taken together, these studies suggest that both PrlA (SecY) and PrlG (SecE) are components of the translocator that interact but perform distinct functions.

H 154 BIOGENESIS OF THE HYDROGENOSOME: AN ENIGMATIC ORGANELLE FOUND EXCLUSIVELY IN PRIMITIVE EUCARYOTES WHICH LACK MITOCHONDRIA. CAROL LAHTI, CHRIS D'OLIVEIRA AND PATRICIA JOHNSON. DEPT. OF MICROBIOLOGY & IMMUNOLOGY, UCLA SCHOOL OF MEDICINE, LOS ANGELES, CA 90024.

The long-term objective of our research is to gain insight into the biogenesis and origin of an unusual organelle found in the parasite Trichomonas vaginalis: the hydrogenosome. Trichomonads are anaerobic protozoan which lack mitochondria yet utilize carbohydrates as a primary energy source. The final steps of carbohydrate fermentation occur in the hydrogenosome. Linked to this process is the production of ATP via substrate level phosphorylation in hydrogenosomes. In this regard, hydrogenosomes could be viewed as the anaerobic equivalent of mitochondria. As an initial step towards studying the biogenesis of this organelle, we have purified stable hydrogenosomes and used these to immunize rabbits. The resulting antisera were used to isolate genes which encode hydrogenosomal proteins. We have focused on two genes, one encoding ferredoxin and the other, succinate thiokinase. Using these genes as probes, we have shown that hydrogenosomal proteins are made on free polysomes. Also, a sequence comparison of the protein and the gene for ferredoxin has provided evidence for a 8 amino acid N-terminal leader sequence on the protein. We are currently developing an in vitro assay for measuring uptake of hydrogenosomal proteins by the purified organelle. This will allow us to determine if the N-terminal extension is cleaved upon translocation, and will lead to the identification of signals which are necessary and sufficient for protein targeting. These data and the implications they provide regarding the catagorization of hydrogenosomes relative to other organelles will be discussed.

H 155 MEMBRANE PROTEINS REQUIRED FOR SECRETION IN *Escherichia coli*.
Kit Johnson, Claudette Gardel, Annick Jacq, Peter J. Schatz, and Jon Beckwith. Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115. The *sec* genes of *E. coli* are required for the proper localization of exported proteins. We have found that the *secD* locus codes for two integral membrane proteins required for secretion, SecD and SecF. The SecD and SecF proteins are similar to one another, both in their primary sequences and in their predicted structure. Preliminary topological evidence suggests that the SecD protein contains a large (40kD) periplasmic domain, and up to six transmembrane stretches. The SecF protein has a much smaller periplasmic domain, but a similar number of predicted transmembrane stretches. The *secE* gene codes for a small integral membrane protein which has three transmembrane stretches. Therefore, of the six identified *sec* genes, four code for integral membrane proteins (*secD*, *secE/prlG*, *secF*, and *secY/prlA*), one codes for a peripheral membrane protein (*secA/prlD*), and one codes for a cytoplasmic protein which is required for the secretion of only a subset of proteins (*secB*). The four membrane proteins may constitute the membrane apparatus for protein translocation.

H 156 IODINE 123 LABELLED STEROIDS FOR NEGATIVE CONTROL OF STEROID RECEPTORS CONTAINING TUMORS.
Marc Zeicher, Pierre Henrot, Jacques Quivy, Micheline Loos*, Pierre Strijckmans*
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In the process of their decay, some radioisotopes release cascades of low Energy Auger electrons possessing a subcellular range of action (a few nm.). If the decay occurs in the vicinity of the DNA it will induce double strands breaks and less than 100 disintegrations are enough to kill a cell. On the contrary if it occurs at the cell surface or within the cytoplasm, it has a negligeable effect on the cell survival. One way to bring these radioisotopes near the DNA is to attach them to steroids with high affinity for the nuclear associated steroid receptor. We have synthesized an estrogen derivative with very high affinity for the estrogen receptor (ER), the Z isomer of the 11ß-chloromethyl-17α-iodovinyl-estradiol (11ßCMIVE). It was labelled with Iodine 123, an Auger electron emitting radionuclide of short half-life (13.21 h). This agent demonstrates a very selective biodistribution in ER. containing tissues. In vitro cytotoxicity was assessed using a modified MTT assay and a clonogenic assay on human cancer cells and normal bone marrow cells. In vivo experiments were performed on nude mice bearing human tumors. A very selective tumor growth inhibition was obtained both in vitro and in vivo. These results indicate that the hormone superfamily of receptors could be suitable targets for radiotherapy of receptors containing tumors.

Protein Sorting and Organelle Retention

H 200 ABLATION OF A SINGLE ASPARAGINE-LINKED GLYCOSYLATION SITE IN THE CELLULAR ISOFORM OF THE PRION PROTEIN PREVENTS ITS TRANSPORT TO THE CELL SURFACE, Mark Rogers, Albert Taraboulos, Michael Scott, Darlene Groth and Stanley B. Prusiner, University of California, San Francisco, CA 94143

The cellular isoform of the prion protein (PrP^C) is a sialoglycoprotein bound almost exclusively on the external surface of the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor. The deduced amino acid sequence of Syrian hamster PrP^C identifies two potential sites for the addition of Asn-linked carbohydrates at amino acids 181-183 (NIT) and 197-199 (NFT). We have altered these sites by replacing the threonine residues with alanines and expressed the mutant proteins transiently in CV1 cells utilizing a mutagenesis vector with the T7 promoter located upstream from the PrP gene. The T7 polymerase was supplied by infection with a recombinant vaccinia virus (Fuerst *et al.*, PNAS 83:8122, 1986). The 3 mutant proteins (PrP_{Ala183}, PrP_{Ala199} and $PrP_{Ala183/199}$) have a reduced relative molecular weight (M_r) compared to wild-type (wt) PrP. Deglycosylation (or synthesis in the presence of tunicamycin) reduced the M_r of all the PrP species to that of the double mutant $PrP_{Ala183/199}$. Our results indicate that both single-site mutant prion proteins are glycosylated at non-mutated sites and they suggest that both potential sites for Asn-linked glycosylation are utilized in wt PrP^C. Immunofluorescent studies demonstrate that while wt PrP localizes to the cell surface, all the mutant PrP molecules accumulate intracellularly. The site of accumulation of PrP_{Ala183} is probably prior to the mid-Golgi stack since this protein does not acquire resistance of Endoglycosidase H. Whether the GPI anchor is added and correctly processed in these mutant PrP molecules remains to be determined.

H 201 A NOVEL SECRETORY PATHWAY FOR INTERLEUKIN 1 ß, A PROTEIN LACKING THE SIGNAL SEQUENCE. Anna Rubartelli, Marina Talio, Adriana Bajetto, Federico Cozzolino* and Roberto Sitia. Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy and *University of Firenze, Italy.

Several proteins with a defined extracellular function have been described which lack a signal peptide able to target them into the classical ER-Golgi secretory pathway, raising the question of how they can leave the producing cell. Among them, we focused our attention to Interleukin 1 ß (IL-1 ß), a soluble mediator of inflammation and immune response. Our data demonstrate that: i) IL-1 ß is selectively released by human monocytes and its secretion is not due to cell death; ii) drugs which block at various levels the export of classical secretory proteins, like brefeldin A or monensin, do not inhibit the secretion of IL-1 ß, indicating that this protein does not follow the classical pathway; iii) conditions which cause cellular stress, like heat shock or treatment with several drugs, increase the secretion of IL-1 ß. Secretion of ADF-thioredoxin, another protein lacking the signal peptide, is also increased by stress conditions, suggesting that members of this novel class of secretory proteins may share the same route of secretion.

Supported by AIRC.

H 202 SEARCHING FOR A SORTING SIGNAL OF THE MHC CLASS II MOLECULE ASSOCIATED INVARIANT CHAIN (Ii).Oddmund Bakke and Bernhard Dobberstein. EMBL, Heidelberg, FRG. The MHC molecules are intracellularily associated with the invariant chain (Ii). This polypeptide has recently been shown to be required for processing/ presentation of native antigen to T helper cells (Stockinger et al. Cell, 56, 683-89, 1989). We have investigated intracellular transport and location of Ii. Ii spans the ER membrane once and exposes its N-terminal on the cytoplasmic side. The assembly of Ii with class II molecules occurs shortly after insertion into the ER membrane. Ii dissociates from the class II molecules in a post Golgi compartment. Preliminary data shows that the invariant chain is found in a post Golgi, mannose-6-phophate receptor rich compartment (Lipp et al., unpublished). Ii must therefore contain sorting information which directs it to this location, or be connected with molecules containing this information. To search for a sorting signal on Ii we have made progressive deletions from the N-terminus and expressed the mutated proteins in CV1 cells. The intracellular location was studied by microscopy. The Ii mutant lacking 11 aa was found in cytoplasmic vesicles like the native protein, whereas deletion of 15 aa resulted in a surface membrane protein. This indicate that intracellular sorting information of Ii is located within the cytoplasmic tail and that aa 12-15 is important for sorting.

H 203 A Subdomain of the Polymeric Immunoglobulin Receptor Cytoplasmic Tail Specifies Basolateral Targeting in MDCK Cells. J.E. Casanova and K.E. Mostov, Dept. of Anatomy, UC San Francisco 94143.

The polymeric immunoglobulin receptor (pIg-R) mediates the transcytosis of IgA and IgM across mucosal epithelia. When cDNA encoding pIg-R is expressed in polarized MDCK cell monolayers the receptor binds and transcytoses IgA as *in vivo*. We have previously shown that the 103 amino acid C-terminal cytolpasmic domain of pIg-R (residues 653-755) is required for correct receptor targeting to the basolateral cell surface as well as for endocytosis. Using site-directed mutagenesis, we have attempted to further localize specific subdomains of the cytoplasmic tail that may mediate these functions. Truncation of the protein at Arg 669 yields a receptor with a 16 amino acid cytoplasmic domain (residues 653-669). This truncated receptor is delivered to the basolateral cell surface, as assayed by protease sensitivity and domain-selective biotinylation, but is not endocytosed. Conversely, an in-frame deletion of residues 655-668, leaving the remainder of the tail intact, yields a receptor that is delivered directly apically rather than basolaterally, and is capable of endocytosis, although at a reduced rate. We conclude that this short segment (655-668) contains a signal that is both necessary and sufficient for basolateral targeting of pIg-R in MDCK cells, and that is topologically distinct from the region involved in receptor endocytosis.

H 204 CHARACTERIZATION OF APICAL AND BASOLATERAL TRANSPORT VESICLES FROM MDCK CELLS. Mark K. Bennett, Angela Wandinger-Ness, Teymuras Kurzchalia*, and Kai Simons. European Molecular Biology Laboratory, 6900 Heidelberg, FRG, *Akademie der Wissenschaften der DDR, Zentral Institute für Molekular Biologie, 1115 Berlin, GDR.

Transport vesicles derived from the trans-Golgi network and destined for the apical and basolateral plasma membrane domains of MDCK cells can be recovered following perforation of the cells with a nitrocellulose filter (Bennett et al., EMBO J., 7:4075). We have used a combination of differential sedimentation and specific immunoisolation techniques to isolate the transport vesicles and define their protein compositions. Two-dimensional gel analysis of vesicles isolated from cells metabolically labeled with ^{35}S-methionine has identified proteins that either fractionate specifically with the apical or basolateral vesicles, or that fractionate with both classes of vesicle. The topology of these proteins and their partitioning during Triton X-114 phase separation has been determined. An alternative method for labeling vesicle proteins was developed. This method takes advantage of a fluorescent lipid marker that can be accumulated in the vesicles (NBD-ceramide and its metabolites). The NBD-labeled lipids can act as a photosensitizer for the membrane-specific photoactivatable probe ^{125}I-iodonapthalene azide (INA; Raviv et al., PNAS, **84**:6103). Transport vesicles were isolated from NBD-ceramide labeled cells and equilibrated with the INA probe. The vesicles were then irradiated at 488 nm (a wavelength that does not directly excite the INA). Two-dimensional gel analysis revealed that only proteins that were previously identified in the ^{35}S-methionine labeled vesicles as being vesicle specific were labeled with INA. Moreover, the INA labeled a subset of the vesicle integral membrane proteins (as determined by Triton X-114 phase partitioning) suggesting that this set of proteins may be in close proximity to the fluorescent lipids.

H 205 EXPRESSION OF RAT RENAL γ-GLUTAMYLTRANSPEPTIDASE (γGT) IN LLC-PK$_1$EPITHELIAL CELLS AS A MODEL FOR APICAL TARGETING. Rebecca P. Hughey, Anne V. Orr, Richard A. Altman and Norman P. Curthoys*, Dept. of Micro., Biochem. and Molecular Biology, Univ. of Pgh. Medical School, Pittsburgh, PA 15261, and *Dept. Biochem., Colorado State Univ., Fort Collins, CO 80523.

Rat renal γGT is a well characterized microvillar hydrolase which is apically expressed in epithelial cells. The propeptide of 62,000 daltons includes an uncleaved signal sequence that serves as the membrane anchor and a cytoplasmic domain of only four residues. The mature γGT contains five N-linked and two O-linked oligosaccharides and is cleaved to yield an amphipathic large subunit and a hydrophilic small subunit. Both the uncleaved and truncated forms of γGT are also expressed *in vivo*. The γGT thus represents a simple endogenous protein which should be an ideal probe of polarized expression.

Multiple cDNA clones encoding the full-length γGT mRNA have been isolated and characterized. While the coding sequences appear identical by restriction mapping, two different 5' noncoding regions have been identified, sequenced and used for Northern blot analysis of RNA from many tissues. The cumulative data is consistent with transcription of the single rat γGT gene from multiple promoters. Both γGT cDNAs have also been expressed in LLC-PK$_1$ (porcine kidney) cells as judged by Northern blot and immunoprecipitation analysis utilizing *rat*-GT specific cDNA probes and IgGs, respectively. Polarized expression will be assessed by growth of cells on Millipore HA filter inserts. Altered forms of γGT are being prepared by oligonucleotide site directed mutagenesis, which either lack site(s) for glycosylation or represent truncated forms of the protein. These mutant γGTs will be similarly studied in LLC-PK$_1$cells in order to characterize the function of the oligosaccharides and the targeting signals for polarized expression.

H 206 IDENTIFICATION OF INTRACELLULAR TARGETING DOMAINS IN POLYPEPTIDE HORMONE PRECURSORS. Dennis Shields, Ann Danoff, Seung Hong, Maureen Cartoon. Albert Einstein College of Medicine, New York, N.Y. 10461. Most small polypeptide hormones (less than 50 amino acids) are synthesized as larger inactive precursors which undergo a series of post-translational modifications to generate a bioactive molecule; these include endoproteolytic processing, glycosylation, amidation, acetylation and phosphorylation. To identify putative intracellular sorting domains in peptide hormone precursors, we have been investigating the heterologous expression of two of the simplest prohormones, namely pancreatic islet prosomatostatins (proSRIF) I and II (Stoller and Shields J.C.B. 107, 2087, 1988; J.C.B. 108, 1647, 1989). When introduced into rat anterior pituitary GH_3 cells, which do not proteolytically process the endogenously expressed growth hormone and prolactin, proSRIF-I is accurately processed to the mature hormone and efficiently targetted to the regulated secretory pathway. In contrast, when proSRIF-II (which shares 50% homology to proSRIF-1) is expressed in GH_3 cells, it is neither proteolytically processed or secreted. Instead, it is quantitatively degraded intracellularly in a chloroquine/NH_4Cl-sensitive compartment, most probably, in lysosomes. Since the major differences between pro SRIF-I and II reside in their NH_2-terminal propeptides, our data suggest that the propeptides possess sorting sequences which enable these precursors to be targeted to different organelles. To identify targetting domains, we constructed chimeric molecules comprising a series of truncated and mutated proSRIF-I molecules fused to α-globin. When native proSRIF-I was fused to α-globin, this chimera was cleaved to generate mature α-globin, of which approximately 25%-35% is targetted to the regulated pathway and secreted. In contrast, the control signal peptide-α-globin fusion is translocated into the E.R. and rapidly degraded (t 1/2 5 min) during transport to the Golgi apparatus. Our results suggest that the propeptides of hormone precursors contain structural information necessary for sorting to the regulated pathway. Supported by grants from NIH and the Lucille Markey Charitable Trust.

H 207 BIOSYNTHESIS OF CARTILAGE CHONDROITIN SULFATE PROTEOGLYCAN INVOLVES THE SEGREGATION OF PRECURSORS WITHIN A SUBCOMPARTMENT OF THE ENDOPLASMIC RETICULUM. Barbara M. Vertel, Angel Velasco, Linda M. Walters, Colleen M. O'Donnell, Paul F. Goetinck, Sherrie LaFrance, and Karen Kaczman-Daniel. Department of Cell Biology and Anatomy, University of Health Sciences/The Chicago Medical School, North Chicago, IL 60064 and La Jolla Cancer Research Foundation, La Jolla, CA 92037. Chondrocytes in culture synthesize several extracellular matrix molecules concurrently. Within individual cells, chondroitin sulfate proteoglycan (CSPG) and type II procollagen display unique cytoplasmic distributions. CSPG precursors are localized primarily in the perinuclear Golgi, while type II procollagen is present in the rough endoplasmic reticulum (ER). In addition, CSPG precursors are observed within a unique cytoplasmic compartment distinct from the perinuclear Golgi. Simultaneous double immunofluorescence localization studies demonstrate that this intracellular compartment contains CSPG precursors not yet modified by Golgi-mediated processes. Link protein, a separate gene product which functions to stabilize extracellular aggregates of CSPG monomer and hyaluronic acid, is colocalized in this compartment, but type II procollagen is excluded. Immunoelectron microscopic analysis utilizing immuno-peroxidase reactions in a preembedment protocol reveals that these CSPG precursors are contained in smooth membrane-bounded regions of the ER continuous with the ribosome-studded ER lumen. Thus, CSPG precursors are segregated within the ER of chondrocytes. The presence of link protein and CSPG monomer precursors and the absence of type II procollagen in these subcompartments of the ER would suggest that the regions play a role in the normal synthesis and processing of cartilage proteoglycans. Chondrocytes from chicken embryos homozygous for the mutation *nanomelia* (defective in the production of CSPG) synthesize a smaller CSPG core protein precursor that does not undergo the posttranslational processing characteristic of the normal cartilage core protein. Immunolocalization studies suggest that the defective precursor accumulates in similar regions of the ER and does not appear to be transported to the Golgi. Other studies to be presented demonstrate that incubation at low temperature and treatment with inhibitors modulate the biosynthesis and cytoplasmic distribution of these matrix precursors. (Supported by NIH grants AM 28433, HD 22016).

H 208 OPPOSITE BUDDING POLARITY OF RNA VIRUSES IN DIFFERENT EPITHELIAL CELLS. Chiara Zurzolo, Claudio Polistina, Marco Saini, Stefano Bonatti and Lucio Nitsch. C.E.O.S., and Dpt. Biol. Patol. Cell. Mol., Napoli, Italy.
Virus budding has been used as a model system to study cell polarity. We show here that budding of two RNA viruses in thyroid epithelial cells is polarized and that it has an opposite orientation with respect to other non-thyroid cells. FRT rat thyroid epithelial cells were cultured in Coon's modified Ham's F12 medium containing 5% FCS; CaCo-2 human colon carcinoma cells were cultured in DMEM containing 20% FCS. Both cell lines were grown to confluency, either on plastic or on filters, and infected with Sindbis Virus (SV) or Semliki Forest Virus (SFV). Budding polarity was assessed by transmission electron microscopy. It was found that both viruses had an apical budding polarity in FRT cells while they had a basolateral budding polarity in CaCo-2 cells. It had been previously determined that SFV had a basolateral budding polarity also in MDCK cells. Since virus budding polarity seems to depend upon polarized delivery to the plasma membrane of virus glycoproteins we determined, by immunoelectronmicroscopy, the distribution of viral glycoproteins on the plasma membrane of FRT cells. It was found that it correlated well with the budding polarity of the two viruses. Preliminary experiments indicate that SV had an apical budding polarity also in reconstituted thyroid follicles in primary suspension culture. We conclude that FRT thyroid cells, with respect to MDCK and CaCo-2 cells, manifest opposite sorting polarity of some RNA viruses and of their membrane glycoproteins. This may be due either to a different post-translational modification of the sorting signal or to some difference in the cell sorting machinery.

H 209 PROCESSING AND TARGETING OF ALEURAIN, A CYSTEINE PROTEASE IN BARLEY ALEURONE CELLS
Barry C. Holwerda, Nancy J. Galvin[1] and John C. Rogers, Dept. of Medicine, Washington University School of Medicine, St. Louis, MO 63110 and [1]Dept. of Pathology, St. Louis University School of Medicine, St. Louis, MO 63104. Aleurain is a cysteine protease closely related to mammalian cathepsin H that was originally characterized as the translation product of a cDNA isolated from barley aleurone layers. We have characterized the protein by use of an antibody made to a bacterial trpE-aleurain fusion protein. Mature aleurain is 32 kDa in size and has one endo H-sensitive high mannose and one complex oligosaccharide side chains as shown by labelling in the presence of tunicamycin. Although aleurone cells are well characterized by their ability to secrete large amounts of hydrolases, aleurain is not secreted. Immunocytochemistry shows that aleurain is located in small vesicular structures that band in 40% sucrose. Pulse chase experiments show that a 42 kDa proform of aleurain is rapidly chased to a 33 kDa form which is then processed to the mature 32 kDa form. Inclusion of tunicamycin in pulse-chase experiments showed that unglycosylated forms of proaleurain are proteolytically processed in a manner similar to native proaleurain. This indicates that oligosaccharides are not required for either processing and/or correct targeting to the site of processing. In pulse chase experiments, the cysteine protease inhibitor E64 prevented the maturation of the 33 kDa to 32 kDa form, but did not interfere with the 42 kDa to 33 kDa conversion. Using as substrate, proaleurain, made by injection of in vitro synthesized aleurain mRNA into Xenopus oocytes, we have identified an activity in crude extracts of aleurone cells that cleaves this to a 33 kDa form. This activity is not inhibited by either leupeptin or E64, but E64 does inhibit the conversion of 33 kDa to 32 kDa forms in vitro.

H 210 ROLE OF THE PROREGION FOR THE PRODUCTION AND SECRETION OF THE *Y. lipolytica* ALKALINE EXTRACELLULAR PROTEASE. Fabre, E., Nicaud, J.M. and Gaillardin C. Laboratoire de génétique, Institut Nationnal Agronomique, 78850 Thiverval Grignon, FRANCE.
The yeast *Yarrowia lipolytica* secretes an Alkaline Extracellular Protease (AEP) which is encoded by the *XPR2* gene. The DNA sequence revealed that it codes for a preproenzyme precursor which presents a putative signal sequence, a stretch of ten X-Ala or X-Pro dipeptides substrates for a dipeptidyl amino peptidase, a proregion presenting a glycosylation site and two Lys-Arg sites possible substrate for a *KEX2* like enzyme and the mature protein. Pulse-chase and immuno-precipitation experiments have confirmed this processing (Ogridziak et col.). In order to determine the role of the proregion we have generated deletions in the proregion, mutated the glycosylation site and inserted linker in the region coding for the mature protease. The results presented indicate that any deletion in the proregion results in intracellular accumulation of the precursor or a toxic effect. Mutation of the glycosylation site or addition of a linker results in temperature dependent secretion and production of active AEP. From these results, we propose that the AEP proregion has a zymogene function and plays an essential role in guiding the proper folding of the protein to allow a conformation which is compatible for secretion.

H 211 INTERACTION OF CHROMOGRANIN B (SECRETOGRANIN I) WITH SECRETORY GRANULE MEMBRANES IN PC 12 CELLS.
Sanjay W. Pimplikar and Wieland B. Huttner, Cell Biology Programme, EMBL, Heidelberg, FRG.
We have been interested in studying how regulated secretory proteins are sorted to secretory granules. In this context, we have investigated the association of Chromogranin B (CgB), a secretory granule protein of most neuroendocrine cells with secretory granule membranes in PC 12 cells. When these cells are stimulated by 50 mM potassium, the secretory granules fuse with the plasma membrane resulting in the release of CgB into the extracellular medium. Using various experimental approaches including immunofluorescence microscopy and cell surface labelling, we show that a small part of the total cellular CgB is tightly associated with the secretory granule membrane and upon stimulation of secretion remains associated with the plasma membrane. Upon subsequent incubation of cells at 37 °C, this cell surface-associated CgB is rapidly internalised. This membrane-associated CgB cannot be extracted with pH 11 treatment but partitions into the aqueous phase upon Triton X-114 phase condensation. It is barely detected on the surface of unstimulated cells or on cells that were incubated with 10 mM ammonium chloride (which is known to cause missorting of CgB to the constitutive pathway of secretion). Moreover, when CgB is added to unstimulated cells, it does not become tightly associated with the plasma membrane. Taken together, these observations suggest that the surface-bound CgB reflects the existence of a subpopulation of CgB molecules that are tightly associated with the secretory granule membrane and, after exocytosis, become exposed at the plasma membrane. The biological significance of this membrane-associated CgB is currently under investigation.

H 212 FORMATION OF IMMATURE SECRETORY GRANULES IN PC12 CELLS, AND IN A CELL-FREE SYSTEM DERIVED FROM PC12 CELLS, Sharon A. Tooze and Wieland B. Huttner, Cell Biology program, EMBL, 6900 Heidelberg, FRG. Specialized cells such as endocrine and neuroendocrine cells have both the constitutive and the regulated pathways of secretion. One morphological hallmark of cells which have the regulated pathway are large dense-core secretory granules. Secretory granules, which contain hormones and other biologically active substances in a concentrated aggregate, form from the trans Golgi network (TGN). The formation of secretory granules has been documented extensively using morphological techniques, however, the molecular mechanisms involved are still largely unknown. To obtain information on the formation of secretory granules we have studied the transport, out of the TGN, of secretogranin I and secretogranin II, two proteins targetted to the regulated pathway, using a combination of sulfate labelling, which labels proteins immediately prior to their exit from the TGN, and subcellular fractionation based on velocity-controlled centrifugation. Using these techniques we have identified the first vesicular intermediate in the biogenesis of large dense-core secretory granules, the immature secretory granule. Immature secretory granules were formed rapidly ($t_{1/2} \approx$ 5 min) from the TGN, and did not contain a constitutively secreted sulfate-labelled proteoglycan. The identification of the first intermediate in the formation of secretory granules has enabled us to develop a cell-free system to dissect the formation process. The cell-free system uses both sulfate labelling and separation of the starting material (TGN) from the product (immature secretory granules) to monitor the formation of immature granules. With this cell-free system we have found that (i) regulated and constitutive proteins are sorted prior to their budding from the TGN in immature secretory granules and constitutive vesicles, respectively, (ii) the budding of both types of post-TGN organelles is inhibited by the addition of GTPγS. We are now investigating the cytosolic and membrane associated factors which may be involved in the cell-free formation immature secretory granules.

H 213 MAXIMIZING SECRETION OF hGHRF FROM SACCHAROMYCES CEREVISIAE, Christine I. Barton and John S. Wood, Molecular Genetics Research, Eli Lilly and Company, Indianapolis, IN 46285

Our goal has been to determine conditions under which maximal amounts of biologically active human growth hormone releasing factor (hGHRF) are secreted from yeast cells and accumulated in the culture medium. We started with a synthetic hGHRF gene, which used yeast codon bias, fused in-frame to the 3' end of the alpha factor signal sequence in a yeast expression vector. Supersecretion mutant yeast strains were transformed with this plasmid construct as well as derivative plasmids containing changes in the 5' control sequences. The effects of these strain and plasmid combinations, as well as variations in media composition and culture conditions, on hGHRF secretion were determined by Western blots and bioassays. We have discovered that the use of our highest level supersecretion mutant, the removal of the glu-ala-glu-ala sequence from the fusion junction, the presence of protease inhibitors in the media during mid-exponential through stationary phase growth, harvesting the culture about 65 hours after inoculation, and the use of rich instead of minimal media result in significantly increased levels of secreted hGHRF.

H 214 REVERSIBLE SECRETAGOGUE-SENSITIVE PHOSPHOGLUCOSYLATION AND SECRETORY RELEASE, R.B. Marchase, J.C. Jay, C. Srisomsap, and L.M. Brumley, Dept of Cell Biology/Anatomy, Univ of Alabama at Birmingham, Birmingham, AL 35294. Treatment of cells exhibiting regulated secretion with secretagogue leads to changes in the phosphate content of several proteins, one of which is a 63 kDa cytosolic phosphoglycoprotein (pgp63) initially described in Paramecium and present in a wide variety of species and vertebrate tissues. Pgp63 appears to exist in two interconvertible states, one containing solely an O-linked Man disaccharide and the other containing, in addition, a phospho-diester-linked terminal Glc transferred enzymatically from UDP-Glc. In digitonin-permeabilized PC-12 cells, the ^{35}S-labeled β-phosphorothioate analogue of UDP-Glc labels pgp63 only when secretion is triggered by μM Ca^{++} and ATP. Other data suggest that in the resting state no acceptor sites are available and that the onset of secretion results first in removal from pgp63 of unlabeled αGlc-1-P by a specific αGlc-1-P phosphodiesterase and its subsequent replacement by the labeled analogue. Further support for this interpretation was obtained when intact PC-12 cells were pulsed briefly with (^{14}C)Glc. Depolarization or secretagogue treatment led to enhanced incorporation of phosphodiester-linked (^{14}C)Glc into pgp63. A similar depolarization-dependent labeling was seen in rat synaptosomes. These results suggest that phosphate donors other than ATP are utilized to reversibly modify proteins in response to stimuli and, in particular, that a cytosolic phosphoglycoprotein may be important in the regulation of secretion.

H 215 SYNAPTOPHYSIN AND P65 ARE DIFFERENTIALLY DISTRIBUTED AND REGULATED IN PC12 CELLS. James J. Lah and Richard W. Burry. Department of Anatomy, Ohio State University College of Medicine, Columbus, Ohio 43210

The functional compartmentalization of neurons poses interesting questions concerning mechanisms of protein sorting and organelle biogenesis. Distal axons lack protein synthesizing machinery, but it is not clear whether organelles such as synaptic vesicles are fully formed in the cell body or if they are assembled from component elements in the periphery. We have begun to address these questions by studying the distribution and regulation of synaptophysin and p65, two integral membrane proteins found in small, clear vesicles of neurons and neuroendocrine cells. In PC12 cells, some of the fluorescent staining for synaptophysin appears to be coincident with wheat germ agglutinin (WGA) binding sites in double labeling experiments. By contrast, p65 staining appears to be diffusely distributed and does not co-localize with WGA. In addition, western blot analysis indicates that stimulation of PC12 cells with nerve growth factor increases the level of p65 while decreasing the level of synaptophysin. These results suggest that the synthesis of these two proteins may be differently regulated; furthermore, the immunocytochemical evidence suggests that they may not be co-localized during early stages of vesicle biogenesis. Funding provided by NSF #BNS-8909835 (RWB) and the Medical Scientist Program OSU (JJL).

H 216 TARGETING OF A SYNAPTIC VESICLE PROTEIN TO LIGHT VESICLES IN NONNEURONAL CELLS, Adam D. Linstedt and Regis B. Kelly, Dept. Biochem., UCSF, SF, CA 94143.

Synaptic vesicles derive from an endocytotic pathway that may be unique to neuronal cells. However, this pathway may overlap with or be an adaptation of a pathway present in all cells. Sequences required for targeting proteins to synaptic vesicles may allow targeting to this precursor pathway in non-neuronal cells. To test this model fibroblast and epithelial cell lines producing a synaptic vesicle specific membrane protein, synaptophysin, were generated by transfection. Immunofluorescence microscopy yielded an unusual "vesicular" punctate staining of synaptophysin throughout the cytoplasm. Indeed, a light vesicular fraction, density equal to synaptic vesicles, comprising approximately 50% of synaptophysin immunoreactivity could be isolated by subcellular fractionation. Analysis by velocity gradient sedimentatation demonstrated that this vesicular population is larger and more heterogeneous in size compared to synaptic vesicles. The mannose-6-phosphate receptor did not codistribute with synaptophysin on velocity gradients. Experiments with the transferrin receptor are in progress. Thus synaptophysin accumulates in a vesicular compartment of nonneuronal cells that is likely to be related to the synaptic vesicle pathway. Further studies should reveal whether these vesicles are part of a known or novel pre-existing pathway or a synaptophysin induced pathway.

H 217 GENETICALLY-MODIFIED SECRETORY TRAFFICKING OF YOLK PROTEINS IN *DROSOPHILA*. Frank Butterworth, Biological Sciences, Oakland University, Rochester, MI 48309 and Mary Bownes, Molecular Biology, University of Edinburgh UK EH9 3JR. The relationship of the amino acid sequence of a secretory protein to its traffic through subcellular compartments is being studied using genetic, molecular and ultrastructural techniques. Molecular studies show that normally yolk protein is synthesized in the fat body, secreted into the hemolymph, and taken up by the ovary. A second site of synthesis is the ovarian follicle cells, where the yolk proteins are probably secreted into the interfollicular spaces and then sequestered by the oocyte. Females homozygous for the mutation *fs(1) 1163* synthesize yolk proteins but do not secrete them and as a result are sterile. The mutation causes an isoleucine to asparagine substitution at position 92 resulting in the triplet Ser Gly Asn^{92}. Ultrastructural studies show twice as many Golgi regions in the fat body of *1163* females, and some cells have massive accumulations of electron-dense granules that often coalesce into tubules associated with smooth endoplasmic reticulum, coated pits and vesicles. In addition, these masses are found in the extracellular, subbasement-membrane space of the cell. Females made transgenic for the *1163* gene with a promoter fragment leading only to fat body expression are fertile but still possess the above subcellular abnormalities. These findings are consistent with the hypothesis that the failure of yolk secretion is due to the creation of a putative glycosylation site which has caused a shunting to the lysosomal pathway in most cells and an aborted secretory path as well in others. Supported by Research Corporation Grant C-2631 (FB) and MRC Project Grant (MB).

H 218 MOLECULAR PROPERTIES OF SYNAPTOPHYSIN, A CHANNEL PROTEIN OF THE SYNAPTIC VESICLE MEMBRANE, Knaus, P., Center for Molecular Biology, University of Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, FRG

Regulated release of neurotransmitter from presynaptic nerve terminals is a process central to brain function. The most widely accepted model of triggered quantal release implies the fusion of synaptic vesicles with the presynaptic plasma membrane. Specific proteins of vesicles as well as the plasma membrane are likely to be involved in mediating and regulating exocytosis.

Synaptophysin is an integral membrane protein of presynaptic vesicles. It shares not only the transmembrane topology with gap junction channel proteins, but also its quaternary structure. Synaptophysin is a homohexameric protein with a diameter of approxiamately 7.8 nm. An extended cytoplasmic domain of the protein is capable of binding calcium ions. Purified synaptophysin, when reconstituted to planar lipid bilayers, produces a large voltage-sensitive channel with multiple conductance levels (unit conductance 150pS in physiological salt solutions). Synaptophysin may be involved in regulating the storage contents of vesicles or may participate in the formation of a pore between the vesicle membrane and the presynaptic terminal to initiate neurotransmitter release.

H 219 BIOGENESIS OF XENOPUS VG1: A NON-SECRETED GLYCOPROTEIN ENCODED ON A LOCALISED MATERNAL mRNA. Alan Colman, Les Dale and Glenn Matthews, School of Biochemistry, Birmingham University Birmingham B15 2TT, England

Vg1 is a glycoprotein with homology to transforming growth factor β which is encoded on a maternal mRNA localised to the vegetal cortex of Xenopus oocytes. Despite the regionalisation of the mRNA the protein product diffuses throughout the oocyte, probably within the ER, however it is not secreted. N-terminal amino acid analysis shows that the signal sequence is not removed, whilst transfection experiments using COS cells, indicate that the protein is retained within the ER. In the oocyte most Vg1 molecules contain 3 mannose-rich oligosaccharide side chains, but during early development a 2-chain form becomes the predominant newly-synthesised Vg1 protein made from the endogenous maternal mRNA. Overproduction of the protein in the embryo can be effected by injection of synthetic mRNA. Under these circumstances the fully glycosylated form predominates. This and other data suggest the emergence in the embryonic secretory pathway of a saturable quantity of a Vg1-binding protein which can influence the degree of Vg1 glycosylation. An *in vitro* assay for the analysis and cloning of this protein has been developed.

H 220 RATES OF GLYCOLIPID TRANSPORT FROM GOLGI TO CELL SURFACE, Felix Wieland, Institut fur Biochemie I der Universitat Heidelberg, Im Neuenheimer Feld 328, D-6900 Heidelberg, FRG. Shortened "truncated" ceramide analogues were added to CHO-cells. The analogues diffuse into cells and serve as precursors for the in vivo formation of truncated sphingomyelin and truncated glucocerebroside, which are then secreted into the medium. Surprisingly, transport rates from their origin (the Golgi) to the plasma membrane of truncated glucocerebroside and truncated sphingomyelin differed markedly. The glucocerebroside analogue showed a half time of secretion of ≈ 7 min., whereas the shortened sphingomyelin was secreted with a half time of ≈ 15 min.

H 221 APOLIPOPROTEIN B IS BOTH INTEGRATED AND TRANSLOCATED ACROSS THE ENDOPLASMIC RETICULUM MEMBRANE: EVIDENCE FOR TWO FUNCTIONALLY DISTINCT POOLS. Roger A. Davis, Tom Sand, Christine C. Wu and Kathryn E. Howell, University of Colorado Medical School, Denver, CO 80262 Previously, we found that only a fraction of de novo synthesized apo B is secreted, the remainder is retained in the endoplasmic reticulum, where it is degraded. To understand the basis for these observations, we examined the first step in the secretory pathway: translocation. Translocation of apo B was determined by its sensitivity to degradation by the exogenous protease: trypsin. Trypsin reduced the amount of apo B in rough microsomes, whereas it had little (if any) effect on degradation of apo B in Golgi fractions. Essentially all of the apo B that was degraded was membrane bound. Monoclonal IgG's, against either the N-terminal or C-terminal halves of apo B, were bound to magnetic beads and used to immuno-isolate microsomes. In contrast, to the specific ability of the IgG's against apo B to isolate microsomes, little or no microsomes were isolated using non-immune IgG and IgG against albumin. Since microsomes remained intact and oriented right side out as demonstrated by the inability of trypsin to both degrade albumin and to affect the capacity of the intraluminal enzyme glucose-6-phosphatase to dephosphorylate mannose-6-phosphate, the data suggest that a pool of apo B is exposed on the cytoplasmic surface of the endoplasmic reticulum membrane. To determine if the trypsin accessible pool of apo B is a transient form, pulse/chase experiments were performed. The results show that the % of apo B that was trypsin accessible increased during the first 20 minutes of the chase, suggesting that during this time, the trypsin accessible pool of apo B is not translocated (it does not become trypsin insensitive). The combined data support the concept that apo B translocation across the endoplasmic reticulum determines its entry into two functionally distinct pools: the intraluminal trypsin insensitive pools in the assembly of VLDL; the trypsin accessible, non-translocated pool is shunted into the degradative pathway. Regulated on of apo B may provide a unique mechanism with which to determine VLDL assembly/secretion.

H 222 AN HDL DEFICIENCY SYNDROME RESULTING FROM DEFECTIVE *trans* REGULATION OF A POST-TRANSCRIPTIONAL STEP IN APOLIPOPROTEIN-A1 EXPRESSION. Alan D. Attie, Ferry Poernama, and Sandra A. Schreyer, Department of Biochemistry, University of Wisconsin, Madison, WI 53706

Apolipoprotein-A1 (apo-A1) is the predominant protein constituent of high density lipoprotein (HDL). HDL deficiency syndromes are strongly correlated with risk of coronary heart disease. In the majority of HDL deficiency cases, the structure of the apo-A1 gene is normal and the causative mutation remains unknown. We discovered a mutation in chickens associated with a ten-fold deficiency in plasma apo-A1. The mutation is sex-linked; the phenotype segregates with markers on the z chromosome. By following the inheritance of an apo-A1 RFLP (in an irrelevant chicken strain) we showed that the apo-A1 gene is autosomal, hence the mutation responsible for the HDL deficiency is *not* in the apo-A1 gene. The levels of apo-A1 mRNA in the mutant animals are not reduced. The translational competence of the apo-A1 mRNA was demonstrated in a reticulocyte lysate system. Nevertheless, the amount of apo-A1 protein mass in liver from the animals is greatly reduced. It therefore appears that the mutants have a defect in a factor regulating an event occuring after transcription and RNA processing. Since apo-A1 is a secretory protein and therefore not exposed to cytoplasmic mechanisms of protein degradation, we think it most likely that the mutation affects translation or the transport of apo-A1 through its secretory pathway.

H 223 FUNCTIONAL ANALYSIS OF SIGNALS FOR PROTEIN RETENTION IN THE PLANT ENDOPLASMIC RETICULUM, Jürgen Denecke, Bart Hellemans and Johan Botterman, Plant Genetic Systems N.V., J. Plateaustraat 22, 9000 Gent, Belgium

We analyzed the requirements for retention of proteins in the lumen of the endoplasmic reticulum (ER) of tobacco cells. Leaf protoplasts and the nonspecific marker enzyme phosphinothricin acetyl transferase (PAT) were used as model system. Previous studies indicated that PAT is secreted by plant cells via a default pathway when translocated to the lumen of the ER (Denecke et al., submitted). Chimeric coding sequences of several PAT derivatives containing potential ER retention signals were fused to the signal sequence of the pathogenesis related protein PR1b and placed under control of the CAMV35S promotor. After introduction in protoplasts by electric field mediated DNA transfer, synthesis and transport of the gene products was analyzed. The canonical sequences KDEL and HDEL, responsible for retention of luminal ER proteins in mammalian cells and yeast, were shown to mediate effective retention of PAT in the lumen of the ER when linked to the C-terminus of the protein. The impact of different mutant signals and the influence of increasing gene expression levels on the efficiency of retention was also analyzed.

H 224 DEFINING THE CONSENSUS SEQUENCE RESPONSIBLE FOR THE RETENTION OF TRANSMEMBRANE MOLECULES IN THE ENDOPLASMIC RETICULUM
Michael R. Jackson, Tommy Nilsson and Per A. Peterson, Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037.
We have recently shown (Nilsson et al, Cell 58, 707-718) that transplanting the cytoplasmic tails of ER resident proteins onto a reporter protein, CD8 (ordinarily expressed on the cell surface), is sufficient to cause retention and accumulation of this protein in the ER. This system has allowed us to test the ability of many different sequences derived from the cytoplasmic tails of resident and non resident ER proteins for their capacity to retain this marker protein in the ER. This data along with that obtained by extensive site directed mutagenesis of these sequences has shown the absolute requirement for lysine residues in the ER retention motiff, in addition these residues must be correctly positioned with respect to the carboxyterminus in order to function efficiently.

H 225 CARBOXYL-TERMINAL PROTEIN SEQUENCES OF SARCOPLASMIC RETICULUM (SR) PROTEINS,
Marek Michalak, Larry Fliegel, Elizabeth Newton and Kimberly Burns, Cardiovascular Disease Research Group, Dept. of Pediatrics and Biochemistry, Univ. of Alberta, Edmonton, Canada. The COOH-terminal four amino acids of several peripheral membrane proteins of endoplasmic reticulum (ER) are important in their retention within the ER lumen. In the heart and skeletal muscle, a number of peripheral SR membrane proteins have recently been identified. Calreticulin is a 55-kDa Ca^{2+} binding protein, contained within skeletal and heart muscle SR and non-muscle ER. We isolated a cDNA clone encoding for calreticulin from a neonatal rabbit skeletal muscle λgt11 library. The deduced COOH-terminal amino acid sequence was KDEL - identical to the retention signal of the ER proteins. The signal probably encodes for retention of this protein within the SR lumen. A 55-kDa thyroid hormone binding protein, which is probably identical to the protein disulfide isomerase, β-subunit of prolyl 4-hydroxylase and glycosylation site binding protein of oligosaccharyl transferase, is also present within muscle SR. Analysis of a cDNA clone from slow-twitch rabbit skeletal muscle encoding this protein showed that it contained a similar but not identical COOH-terminal sequence of RDEL. This suggests that the positively charged amino acids K and R can interchange and the retention signal is still active. In contrast to these results calsequestrin and a 53-kDa glycoprotein, two other peripheral membrane proteins residing in the lumen of the SR, do not contain the KDEL retention signal. The SR may have developed a unique retention mechanism(s) for these muscle specific proteins.

H 226 A PEPTIDE SEQUENCE CONFERS THE PHENOTYPE OF RETENTION AND RAPID DEGRADAT WITHIN THE ENDOPLASMIC RETICULUM,
Juan S. Bonifacino, Carolyn K. Suzuki and Richard D. Klausner, Cell Biology and Metabolism Branch, NICHD, National Institutes of Health, Bethesda, MD 20892.
Recent studies have demonstrated the existence of a nonlysosomal pathway for the degradation of newly synthesized proteins retained within the endoplasmic reticulum (ER). This pathway is extremely selective, in that whereas some proteins are rapidly degraded, others survive for long periods in the ER. The question of whether this selectivity is due to the presence of peptide sequences or structural motifs within the sensitive proteins that target them for degradation has been addressed. We initially searched for such sequences by dissecting domains of the alpha chain of the T cell antigen receptor (TCR-α), a protein that is retained and degraded within the ER when expressed in fibroblasts. Deletion of a 28-amino acid carboxyl terminal sequence, comprising the transmembrane domain and short cytoplasmic tail of TCR-α prevented the rapid degradation of this polypeptide. Fusion of this carboxyl terminal sequence to the extracellular domain of the alpha chain of the interleukin-2 receptor (also known as Tac antigen) a membrane protein that is normally transported to the cell surface where it survives long term, resulted in the retention and rapid degradation of the chimeric protein in the ER. Additional mutagenesis revealed that the transmembrane domain of TCR-α alone was sufficient to cause degradation within the ER. Blocking transport out of the ER system with brefeldin A did not lead to degradation of the Tac antigen, indicating that degradation of the chimeric proteins was not a direct consequence of retention in the ER. These observations suggest that a 23 amino acid sequence, including the transmembrane domain of TCR-α, contains information that determines degradation within the ER system.

H 227 BASIS FOR β-GALACTOSIDE α2,6-SIALYLTRANSFERASE LOCALIZATION TO THE GOLGI APPARATUS. Karen J. Colley, Eryn Ujita Lee, *Beverly Adler, *Jeffrey K. Browne, and James C. Paulson. Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024-1737 and *AMGen, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320. In order to investigate the basis for the retention of the β-galactoside α2,6 sialyltransferase (ST) within the Golgi apparatus, we have expressed and localized both wild type and mutant forms of the ST in Chinese hamster ovary and Cos-1 cells. The presence of soluble glycosyltransferases in body fluids suggests that some proportion of glycosyltransferases are released from their membrane anchors by proteolysis. The observation that proteolysis occurs during the purification of the ST from rat liver and results in a 41 kDa form which begins with amino acid 63 of the intact, 47 kDa, Golgi form of the enzyme indicated that some or all of the first 62 aa of the ST, which include a 9 aa, NH_2-terminal cytoplasmic tail, a 17 aa signal-anchor domain and an extended stem region, may be responsible for the retention of the ST in the Golgi. To directly test this, we replaced the first 57 aa of the ST with the cleavable signal peptide of human γ interferon and expressed this signal peptide-sialyltransferase (sp-ST) fusion protein and wild type ST in CHO cells. While the wild type ST was retained within the cells, the signal peptide was cleaved from the sp-ST fusion protein, resulting in the secretion of a soluble, catalytically active enzyme ($t_{1/2}$=2-3 hours). In addition, the wild type ST cDNA was altered by oligonucleotide-directed mutagenesis to contain a signal peptide cleavage site at the COOH-terminal end of the signal anchor domain. Transient expression of this signal cleavage-ST (sc-ST) in Cos-1 cells demonstrated that this altered enzyme was secreted very slowly from the cells ($t_{1/2}$ >24 hours) and the sc-ST enzyme remaining within the cells was predominantly localized to the Golgi apparatus by immunofluorscence. Another mutant, Δtail-ST, which is lacking most of the cytoplasmic tail, behaves like the wild type ST and is localized to the Golgi apparatus in the Cos-1 cells. These preliminary results suggest that the NH_2-terminal stem region contains the "signal" required for ST retention within the Golgi apparatus. (Supported by NIH grants GM 27904 and GM 11557).

H 228 THE ER RETENTION "RECEPTOR" OF THE E3/19K PROTEIN OF AD-2, Björn Dahllöf, Reinhard Gabathuler, Margareta Wallin* and Sune Kvist, Ludwig Institute for Cancer Research, Karolinska Institute, Stockholm, S-104 01, *Department of Zoology, University of Göteborg, S-400 33, Sweden.

The E3/19K protein of adenovirus type 2 is a resident of the endoplasmic reticulum (ER). Three segments of amino acids in the cytoplasmic tail constitute the retention signal. Here we show that the same amino acids are involved in binding to tubulin. A peptide, p34, corresponding to the cytoplasmic carboxy-terminus of the E3/19K protein, stimulates microtubule-protein polymerization and can also induce tubulin polymerization in the absence of microtubule-associated proteins (MAPs). A high degree of similarity is found between the tubulin-binding repeats of two MAPs and p34. By using peptide variants of p34 we could show that the three amino acid segments necessary for retention of the E3/19K protein coincide with residues needed to induce tubulin-binding and polymerization. Immunofluorescence experiments using antibodies against the C-terminus of the E3/19K protein gave rise to a filament-like pattern in cells transfected with the E3/19K gene. These filaments were found to be localized to microtubules, as revealed by double-staining. Depolymerization of microtubules by nocodazole leads to transformation of the "E3/19K filaments" into thicker structures. Similar E3/19K structures were also found in untreated mitotic cells. We suggest that the mechanism for ER retention of the E3/19K protein is mediated by its strong association to the microtubule network.

H 229 INTRACELLULAR RETENTION OF THE E1 GLYCOPROTEIN FROM AN AVIAN CORONAVIRUS INVOLVES THE FIRST MEMBRANE-SPANNING DOMAIN. Carolyn Machamer and Ann M. Swift, Department of Cell Biology & Anatomy, Johns Hopkins Medical School, Baltimore, MD 21205

We are using the E1 glycoprotein from the avian coronavirus infectious bronchitis virus as a model protein for studying intracellular retention of membrane proteins. The E1 protein is targeted to the Golgi complex in animal cells transfected with cloned cDNA. Previous experiments involving large deletions of E1 (Machamer and Rose, *J. Cell Biol.* 105:1205-1214, 1987) showed that the first of the three membrane-spanning domains (m1) may be required for this intracellular retention. Using oligonucleotide-directed mutagenesis, we have produced single amino acid substitutions in m1 to ask if more subtle changes can disrupt the retention signal and result in transport of E1 to the plasma membrane. These changes were introduced into the wild-type E1 protein, as well as the deletion mutant Δm2,3 which lacks the second and third membrane-spanning domains. We find that several substitutions (hydrophobic for uncharged polar residues) and a small insertion (two isoleucine residues) in the m1 domain result in transport of Δm2,3 to the plasma membrane. However, these same changes in the full-length E1 protein do not result in transport to the plasma membrane, and in fact appear to hinder transport out of the endoplasmic reticulum. These results suggest that m1 may contain a signal for retention in the Golgi (at least for Δm2,3), and may also influence the proper folding of the wild-type E1 protein. We are currently addressing the mechanism by which these proteins are retained intracellularly.

H 230 CHARACTERIZATION OF AN INTRACELLULAR RECEPTOR FOR THE -KDEL E.R. RETENTION SIGNAL

David Vaux, John Tooze and Stephen Fuller, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany.

The carboxy terminal sequence -KDEL is necesary and sufficient for the retention of soluble proteins within the lumen of the mammalian endoplasmic reticulum (ER). We have identified a candidate protein which may be the receptor for this signal using anti-idiotype antibodies. Two distinct monoclonal anti-idiotype antibodies raised against separate anti-KDEL-peptide (idiotype) antibodies both recognise a 72kD transmembrane protein present in the lumen of the secretory pathway. Rabbit anti-KDEL antisera were found to contain a specific public idiotope recognised by both anti-idiotype monoclonals. Anti-anti-idiotype antisera, generated by immunization of syngeneic mice with fixed anti-idiotype hybridoma cells, recognise a number of -KDEL terminated peptides but not those with -KDELGL termini. By immunofluorescence the antigen is found in a fine cytoplasmic vesicular compartment, which shows perinuclear coalescence in response to a 15°C block, consistent with a putative recycling retrieval role. The putative receptor has been isolated from anti-idiotype hybridoma cells and found to bind to -KDEL terminated ligands in a [Ca^{++}] and pH dependent manner, but not to the ineffective sequence, -KDELGL. The anti-idiotype secreting hybridoma cells maintain wild type levels of soluble ER proteins and do not leak large amounts of these proteins. They appear to survive by sharply upregulating the expression of the putative receptor. Further results show that the anti-idiotype antibodies recognise a highly conserved (from S. pombe to man), physiologically relevant, -KDEL recognition protein with an intracellular location consistent with the role of a recycling receptor for the retention of soluble ER proteins. The anti-idiotype reagents and antisera to the isolated protein are now being used to dissect the molecular basis for the retention of soluble resident proteins in the ER.

H 231 **PROLYL 4-HYDROXYLASE: STEADY-STATE RNA LEVELS OF THE ALPHA-SUBUNIT CORRELATE WITH CHANGES IN COLLAGEN RNA LEVELS DURING CELL GROWTH.**

James A. Bassuk and Richard A. Berg. Department of Biochemistry, Robert Wood Johnson Medical School, University Medicine and Dentistry of New Jersey, Piscataway, NJ 08854.

Prolyl 4-hydroxylase is a key endoplasmic reticulum (ER) enzyme required for the posttranslational hydroxylation of proline residues in the collagen family of proteins. This hydroxylation is required for the folding, translocation, and secretion of collagen. The enzyme consists of a tetramer composed of two pairs of non-identical subunits ($\alpha_2\beta_2$). The β-subunit has been shown to be protein disulphide isomerase, an ubiquitious enzyme found in the ER of many cell types. We recently have determined the amino acid sequence of the chicken α-subunit (Bassuk et al., *Proc. Natl. Acid. Sci. USA 86*: 7382-7386). The mature α-subunit is comprised of 516 amino acids with a calculated molecular size of 59,373 kDa. The compiled amino acid sequence contains two potential glycosylation sites, an observation which agrees with a previous demonstration that the α-subunit contains two N-linked oligosaccharide chains. The polypeptide lacks known C-terminal ER retention signal peptides. In this study, we asked whether the steady-state RNA levels of each prolyl 4-hydroxylase subunit mirrored the changes in collagen RNA levels. Chick tendon fibroblasts were grown in culture for up to 7 days under conditions where increased collagen mRNA synthesis parallels cell density. RNA was prepared each day and analyzed by slot-blot hybridizations. The relative steady-state levels of α-subunit mRNA were correlated with increases of the steady-state levels of collagen mRNAs. The β-subunit mRNA levels and ribosomal protein mRNA levels appeared constant over the 7 days. Since the expression of the α-subunit is confined to cell types that synthesize and secrete collagens, the regulation of α-subunit expression may play a central role in determining the expression of prolyl 4-hydroxylase activity.

H 232 TRANSCRIPTIONAL REGULATION OF S. CEREVISIAE KAR2 (BiP) GENE, Kenji Kohno*, Karl Normington† Mary-Jane Gething† and Joe Sambrook†, * Inst. Molec. Cell. Biol. Osaka Univ., Suita, Osaka 565, Japan, † Dept. Biochem. Univ. Texas SW Medical Ctr. Dallas, TX 75235

The endoplasmic reticulum (ER) of both mammalian and yeast cells contains one of the major luminal 78 kd protein (BiP, KAR2) that is believed to assist in correct folding and assembly of secretory and transmembrane proteins. Like its mammalian counterparts, yeast BiP mRNA is induced by some types of stress such as tunicamycin (TM), A23187, and accumulation of prefolded and/or precursor proteins in the ER, and further, it is induced by heat shock. To determine which sequences are responsible for these stimuli, we cloned a 1.3 Kb upstream region of KAR2 coding sequence and fused it with DNA encoding β-galactosidase gene. Progressive-deletion analysis of this fused gene showed that about 230 bp XhoI-DraI fragment contains all the information required for accurate transcriptional regulation of yeast BiP and indicated that the presence of at least two regions resposive to these stress stimuli. One is a typical yeast heat shock element (TTCNNGAA) and the other one is unfolded protein-response region. They are functionally independent of each other but together work synergistically for maximum induction.

H 233 PROCOLLAGEN FROM A PATIENT WITH OSTEOGENESIS IMPERFECTA (OI) TYPE II IS RETAINED INTRACELLULARLY AND BINDS BiP. Steven D. Chessler, Gillian A. Wallis and Peter H. Byers, University of Washington, Seattle, WA 98195. Mutations in type I collagen genes often cause intracellular accumulation of the defective molecules. We studied impaired secretion in skin fibroblasts from patients with OI, a heterogeneous group of disorders resulting from mutations in the genes encoding type I collagen. We examined 13 OI cell strains with different abnormalities of type I collagen structure or secretion including point mutations, large deletions and altered ratios of chain synthesis and found one strain (88-251) in which there are increased amounts of a protein that comigrates with BiP by two dimensional NEPHGE/SDS-PAGE. In this strain, a point mutation in one COL1A1 allele results in a glycine to serine substitution at triple helical residue 964 in α1(I). BiP accumulation during a 2 hour labelling period peaks about 20 hours after the addition of ascorbate to the culture medium and returns to baseline by 48 hours. This effect, observed even when post-translational procollagen modification is inhibited, is probably due to increased procollagen synthesis. BiP message levels are unchanged by ascorbate treatment. Immunoprecipitation of type I procollagen shows that BiP binds to the abnormal procollagen. Maximal intracellular accumulation of type I procollagen in strain 88-251 is reached within 20 hours with continuous ascorbate treatment. Between 24 and 48 hours, intracellular procollagen levels decrease markedly. Procollagen secretion increases continuously between 5 and 48 hours. In OI strains used for comparison, intracellular procollagen levels and secretion do not change significantly between 5 and 48 hours. These results suggest that BiP accumulates in strain 88-251 fibroblasts in response to increased procollagen synthesis and intracellular procollagen accumulation. BiP may be stabilized by binding to defective procollagens and may enable their secretion. Once the pool of BiP molecules has reached sufficient size, procollagen no longer accumulates, and consequently BiP stops accumulating as well. Since other OI cell strains we have studied, including one with a glycine to serine mutation at residue 1003, exhibit neither BiP binding to type I procollagen nor increased BiP levels, BiP seems to recognize only certain defective procollagens, depending on the mutation and its location. This suggests that there is more than one cellular mechanism for handling abnormal procollagens.

H 234 BOVINE 70 KD HEAT SHOCK COGNATE PROTEIN: PRIMARY AND TERTIARY STRUCTURE, Camille DeLuca-Flaherty, Kevin M. Flaherty, and David B. McKay, Department of Cell Biology, Stanford University Medical Center, Stanford, CA 94305.

There is evidence that the constitutively expressed eukaryotic 70KD heat shock cognate proteins, HSC70's, stimulate post-tranlsational import of precursor polypeptides into mitochondria and the lumen of the endoplasmic reticulum (1). Additionally, the bovine HSC70 has been shown to disassemble clathrin cages in vitro (2). We have initiated structure-function studies on the bovine HSC70 protein. Specifically, we have crystallized the 45 KD amino-terminal ATPase fragment of the protein, and are currently building a molecular model of its x-ray crystallographic structure, using data to 2.9 A resolution. In addition, we have cloned and sequenced the cDNA of the protein, and have found a surprising conservation with the rat HSC70 protein: the amino acid sequences differ in only four positions. Further, expression of a recombinant HSC70 protein and studies on its activity are in progress.

(1) Deshaies, etal., Nature 322:800-805 (1988); Chirico et al., Nature 322:805-810 (1988). (2) Chappell, et al., Cell 45:3-13 (1986).

H 235 Secretory proteins which associate with GRP78 exhibit larger ATP requirements for secretion. Randal J. Kaufman, Louise C. Wasley, and Andrew J. Dorner. Genetics Institute, 87 Cambridge Park Dr. Cambridge, MA 02140.

Some secretory proteins expressed in Chinese hamster ovary cells, such as wildtype tissue plasminogen activator (tPA) and von Willebrand factor (vWF), exhibit a transient association with GRP78, a resident luminal protein of the ER. In contrast, a small proportion of newly synthesized factor VIII and tPA-3X (a mutant which lacks the 3 N-linked glycosylation sites) exhibits a stable association with GRP78 and is not secreted. We have analyzed the importance for ATP availability in secretion by studying whether CCCP, an uncoupler of oxidative phosphorylation, has a differential effect on the secretion of these proteins. Low concentrations of CCCP inhibited the secretion of tPA-3X. Low concentrations of CCCP also inhibited secretion of factor VIII but not vWF synthesized in the same cell. In the presence of CCCP, the majority of the factor VIII was retained a stable complex with GRP78. As the concentration of CCCP increased, secretion of vWF was also inhibited. These studies signify that different proteins exhibit different ATP requirements for secretion and this requirement correlates with the type of association the protein displays with GRP78.

H 236 REGULATION OF GRP78\BiP DURING INFECTION WITH THE PARAMYXOVIRUS SV5, Stephanie S. Watowich, Robert A. Lamb and Richard I. Morimoto, Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208.

Mammalian cells synthesize several members of the heat-shock protein family under normal growth conditions, which are located in various subcellular compartments. The glucose-regulated protein, GRP78\BiP, is a constitutive component of the endoplasmic reticulum (ER), where it appears to play a role in the processing and transport of secretory and membrane-bound proteins. Transcription of the GRP78\BiP gene is stimulated by conditions which alter normal glycoprotein processing and transport. This correlation between the function of GRP78\BiP and its induction has led to the hypothesis that the activity of GRP78\BiP in the ER may be able to modulate GRP78\BiP transcription via some type of feedback system.

We have studied the expression and activity of GRP78\BiP during paramyxovirus (SV5) infection, a condition which stimulates GRP78\BiP synthesis. GRP78\BiP is transcriptionally activated during SV5 infection, at timepoints well after viral structural proteins are first synthesized. GRP78\BiP specifically interacts with HN, one of the two viral structural glycoproteins. This interaction is detected as soon as HN synthesis is detected, well before GRP78\BiP is transcriptionally activated. The interaction is not dependent upon newly synthesized GRP78\BiP. Our results suggest that the synthesis of HN during SV5 infection may be part of the signal which is inducing GRP78\BiP transcription during infection.

H 237 THE IMMUNO-ISOLATION AND CHARACTERIZATION OF MEMBRANE ENVELOPED INTERMEDIATE SA11 ROTAVIRUS PARTICLES FROM INFECTED CELL MICROSOMES USING AN IMMUNO-MAGNETIC MATRIX, Marianne S. Poruchynsky and Paul H. Atkinson, Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, N.Y. 10461.

Rotavirus matures by budding into the RER where it is transiently membrane enveloped. We have developed a scheme for purifying these membrane enveloped biosynthetic intermediates using sucrose gradient fractionation and assay by electron microscopy (EM). Repeated shearing of the fractions released both mature non-enveloped particles and the immature membrane-enveloped virus particles (IMPs). When an affinity purified rabbit polyclonal antibody directed against the nonstructural rotavirus membrane glycoprotein, NS28, was added to the sheared fractions followed by the addition of a magnetic matrix coated with sheep anti rabbit IgG (Dynal M450), IMPs and membrane forms were seen to be specifically bound to the surface of the matrix when viewed by thin section EM and compared with a preimmune serum control. The viral protein composition of the isolated IMPs was determined by radiolabeling the infected cells with ^{35}S-met and ^{35}S-cys prior to immuno-isolation and subsequently analyzing the solublized IMPs by SDS-PAGE. VP1, VP2, VP4, VP6, VP7 and NS28 were prominently seen. When the cross-linkers, DSP or DTSSP, were applied to the IMPs and the labeled proteins examined in both the reduced and non-reduced state, high molecular weight species were seen by SDS-PAGE for the non-reduced samples with the disappearance of bands where NS28, VP7, VP2 and VP4 migrate. Upon reduction, these bands reappeared. These cross-linked unreduced species are being evaluated by sucrose density sedimentation. It is presently being determined if the IMPs contain both the membrane associated and the viral forms of VP7, which are distinguishable with conformation specific antibodies. Isolation of this enveloped viral intermediate lends itself to the in vitro analysis of the energetic and ionic requirements necessary for the uncoating and rearrangement of viral structural and nonstructural proteins.

H 238 FOLDING, TRIMERIZATION AND TRANSPORT OF INFLUENZA HEMAGGLUTININ. Ineke Braakman, Krystn R. Wagner, Helana Hoover-Litty, Ari Helenius. Dept. Cell Biology, Yale University School of Medicine, New Haven, CT 06510.

Proteins destined for the plasma membrane and for secretion fold and frequently oligomerize in the endoplasmic reticulum (ER). To analyze the conformational requirements for transport from the ER to the Golgi, we monitored folding, trimerization and transport of the influenza virus hemagglutinin (HA) in radioactively pulsed cells. *Folding* of the 84kD membrane glycoprotein was assayed using conformation specific monoclonal antibodies and nonreducing SDS PAGE which show formation of intrachain disulfide bonds. *Trimerization* was monitored using trimer-specific monoclonal antibodies, gradient centrifugation and protease resistance, and *transport to the Golgi* was determined by the acquisition of endoglycosidase H resistance. Our results showed the following: 1) While initiated in the nascent chain, folding of HA monomers continued for 5-20 min after completed synthesis. 2) The kinetics of folding were different in different cell types. 3) The rate of trimerization was dependent on the rate of folding and on the level of HA expression. A 5-10 fold decrease in HA expression caused a delay in trimerization from 7 to 17 min. 4) Transport was dependent on the rate of folding and trimer formation. The average rate of transport to the medial Golgi in cells expressing high HA levels was 15 min, and in cells with a 5-10 fold lower expression was 19 min. 5) Mutant proteins with additional sulfhydryl groups (obtained from Drs. D. Wiley and J.Skehel) formed interchain disulfides 5-25 minutes after synthesis. Taken together the results indicated that transport of proteins from the ER depends primarily on folding and secondarily on oligomerization.

H 239 THE EXPORT PATHWAY OF THE PRV gB HOMOLOG gII INVOLVES DIMER FORMATION IN THE ENDOPLASMIC RETICULUM AND PROTEASE PROCESSING IN THE GOLGI. M. E. Whealy, A. K. Robbins, and L. W. Enquist, E. I. du Pont de Nemours & Company, CR&D, Wilmington, DE 19880-0328.
Our laboratory has been studying the envelope glycoproteins of the swine herpesvirus, pseudorabies virus (PRV). The PRV gII glycoprotein shares significant homology with the gB gene of HSV-1. Unlike gB, however, gII is processed by specific protease cleavage events after synthesis of the precursor. The processed forms of gII are maintained in a complex that includes disulfide linkages. In this report, we demonstrate the kinetics of complex formation and subsequent protease processing. In particular, we show that gII dimer formation in the endoplasmic reticulum is an integral part of the export pathway and that protease cleavage occurs after dimers have formed. Furthermore, through the use of glycoprotein gene fusions between the gIII glycoprotein and the gII glycoproteins of PRV, we have mapped the functional cleavage domain of gII to an eleven amino acid segment.

H 240 GENETIC ANALYSIS OF A HERPESVIRUS GLYCOPROTEIN TRANSMEMBRANE DOMAIN. Kimberly A. Solomon, Alan K. Robbins, Mary E. Whealy and Lynn W. Enquist, E.I. du Pont de Nemours Company CR+D, Wilmington, DE 19880-0328. The genome of the swine herpesvirus, pseudorabies virus (PRV), encodes at least seven different viral glycoproteins. Our laboratory has been studying the synthesis, processing and localization of the nonessential envelope glycoprotein, gIII. The predicted primary amino acid sequence of gIII reveals a significant hydrophobic domain (24 amino acids in length) at the carboxy-terminus of the protein characteristic of a membrane spanning or transmembrane region. In this study we use a genetic approach to generate defined mutations within this region in order to examine the role of this domain in gIII export and assembly into virus particles. Six different mutations were constructed which included: 1) a deletion of 19 hydrophobic amino acids, 2) replacement of 20 amino acids with a polyleucine tract, 3) insertion of a nonsense codon in the middle of the transmembrane domain, 4) insertion of a frame shift mutation at the same position, 5 and 6) insertion of 4 or 6 amino acids. These mutations were constructed on bacterial plasmids and crossed onto the PRV genome by co-transfection with viral DNA. Recombinant viruses were identified by using the black plaque immunoassay. Immunoprecipitation of infected cell extracts, virions and media from mutant infected cells revealed different localization phentotypes for the mutants. The role of the gIII carboxy-terminal hydrophobic domain in membrane anchoring and protein localization will be discussed.

H 241 BIOGENESIS OF THE CORONAVIRAL PEPLOMER, Peter J.M.Rottier, Gert-Jan Godeke, Harry Vennema and Willy Spaan, Institute of Virology, Veterinary Faculty, State University, Utrecht, The Netherlands
An interesting aspect of coronaviruses is their intracellular morphogenesis. The viruses assemble at internal membranes by budding into compartments of the ER/Golgi region. The viral membrane proteins M and S accumulate in this area of the cell thereby providing the matrix with which the nucleocapsid can interact.
We have studied the biogenesis and maturation of the peplomer of mouse hepatitis virus strain A59 (MHV-A59). We have found that the spike protein S is synthesized as a gp150 precursor which initially occurs as a monomer but which subsequently dimerizes. Dimerization is a rather slow process (monomer half-life 40-50 min) occurring while the protein is still in an endo H sensitive form. The protein is then incorporated into virions and transported through the Golgi complex and to the plasma membrane. During this process the oligosaccharides become endo H resistant and the protein is cleaved prior to release from the cell.

H 242 MEMBRANE GLYCOPROTEIN COMPARTMENTALIZATION: TARGETING OF HYBRID GLYCOPROTEINS TO NUCLEAR MEMBRANE AND CELL SURFACE. H.P. Ghosh, R. Gilbert, N. Ghosh-Choudhury and D. Snoddy, Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada.
A major focus of this laboratory has been to study the mechanism of tranpsort and sorting of membrane glycoproteins by using enveloped animal viruses which assemble at different intracellular membrane locations. The glycoproteins G of VSV and gB and gC of HSV-1 are targeted to plasma membrane and nuclear envelope inner membrane, respectively, for virus assembly in those locations. The glycoproteins gB and gC of HSV-1 have been expressed from cloned genes in mammalian cells and found to be localized in the nuclear membrane (P.N.A.S., 84, 634 (1987). A number of glycoproteins in which the transmembrane and cytoplasmic domains of gB or gC were exchanged with those of VSV G were constructed and expressed in COS cells. Immunofluorescence studies showed that the chimeric proteins gC-G and gB-G was absent from the nuclear envelope but present in the cell surface. In contrast, the hybrids gBGC and gCGB were localized both in the nuclear membrane and in the cell surface. This data suggests that the transmembrane and cytoplasmic domains may be involved in targeting to nuclear envelope. Further studies using hybrids GGB and GGC are in progress. (Supported by MRC Canada.)

H 243 MATURATION AND TRANSPORT OF THE SIMIAN VIRUS 5 HN GLYCOPROTEIN: SPECIFIC AND TRANSIENT ASSOCIATION WITH GRP78-BiP IN THE ER AND EXTENSIVE INTERNALIZATION FROM THE CELL SURFACE, Davis T.W. Ng, Rick E. Randall*, and Robert A. Lamb, Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208, and *Department of Biochemistry and Microbiology, University of St. Andrews, St. Andrews, Fife KY16 9AL Scotland.
The hemagglutinin-neuraminidase (HN) of the paramyxovirus SV5 is a typical class II integral membrane glycoprotein. It contains an uncleaved 19 amino acid amino-proximal signal-anchor domain which orients the protein in the plama membrane with the large carboxyl-terminal domain in the ectoplasm. The folding, oligomerization, and transport of HN was studied following its synthesis in infected cells. Pulse-chase labeling and sucrose gradient sedimentation analysis indicated that assembly into homotetramers occured with a $t_{1/2}$ of 25-30 min. Conformation-specific monoclonal antibodies to HN were characterized and used to probe folded states during maturation. The resident ER protein, GRP78-BiP, was found to be specifically associated with HN during or shortly following synthesis and dissociated when HN reached a folded conformation just prior to oligomerization ($t_{1/2}$, 20-25 min.). The HN protein also exhibited a high rate of turnover ($t_{1/2}$, 2-2.5 h) that was not attributable to viral budding. It was found that following the transport to the infected cell surface, HN was specifically sorted for endocytosis away from the other SV5-specific membrane glycoprotein, F, and subsequently transported to lysosomes for degradation. The F glycoprotein was found to be relatively stable and remained at the plasma membrane following transport.

Endomembrane Traffic; Endocytosis; Lysosomal and Vacuolar Targeting

H 300 INTERMIXING OF LYSOSOMAL MEMBRANE PROTEINS IN FUSED CELLS IS ACCOMPANIED BY COALESCENCE OF THE PRE-LYSOSOMAL COMPARTMENT (PLC), Brian Storrie, Yuping Deng, and Gareth Griffiths, Biochemistry, Virginia Tech, Blacksburg, VA 24061 and EMBL, Heidelberg, FRG.
We have validated at the ultrastructural level rapid intermixing of resident lysosomal proteins in fused mammalian cells. Comparative stereological measurements were made of volume density of vacuolar compartment and nuclear/cytoplasmic ratio in fused and control cells; no change was observed. To investigate intermixing of lysosomal components, 3T3 and NRK cells were labelled in a pulse-chase protocol (16 h chase) with 16nm and 5nm BSA-gold respectively and then 3 h post cell fusion, UV-inactivated virus- or PEG-mediated, the distribution of fed markers and species-specific lysosomal membrane protein markers characterized using 4 different size gold markers. In cryo-sections, co-localization of fed markers was observed and 82% of the organelle profiles positive for mouse lysosomal membrane protein mLAMP-1 were positive for rat LIMP-I. Triple immunofluorescent labelling for mLAMP-1, LIMP-I and the 215 kDa MPR indicated a scattered, punctate distribution of mLAMP-1/LIMP-I positive lysosomes (MPR^-). PLC was distributed as compact fluorescent foci surrounded by several nuclei. Consistent with PLC fusion in the cell syncytia, a two-fold decrease in cryo-immunogold labelling/μm^2 for MPR and LIMP-I in PLC was observed. To test if fused PLC functioned as one compartment, 3T3-NRK cell syncytia were labelled with rhodamine dextran for 10 min and then incubated in marker-free media for 50 min; extensive overlap of dextran distribution and fused PLC occurred.

H 301 HUMAN LYSOSOME MEMBRANE GLYCOPROTEIN H-LAMP-1 CONTAINS A SIGNAL SUFFICIENT TO DIRECT A HETEROLOGOUS PROTEIN TO LYSOSOMES, Mark A. Williams and Minoru Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA 92037

Site-specific mutations in the COOH-terminal cytoplasmic tail of human lysosome membrane glycoprotein h-lamp-1 cDNA identified a tyrosine residue necessary for efficient lysosomal transport of h-lamp-1 expressed in COS-1 cells. cDNA encoding a chimeric glycoprotein consisting of the hCG-α reporter, the vesicular stomatitis virus glycoprotein transmembrane anchor (G), and the h-lamp-1 cytoplasmic tail (L), designated hCG-α/G/L^{Y}, was also efficiently expressed in lysosomes. Substitution of the tyrosine in this chimeric glycoprotein cytoplasmic tail to make the mutant chimera hCG-α/G/L^{A} eliminated lysosomal expression. These data indicate the signal necessary and sufficient for transport to lysosomes is the short, tyrosine-containing cytoplasmic tail of h-lamp-1. The intracellular distribution of h-lamp-1 cytoplamic tail mutants which were not transported to lysosomes was investigated. The carbohydrate portions of these glycoproteins became resistant to endoglycosidase H, indicating transit through the Golgi. Indirect immunofluorescence of the surfaces of live, intact transfected cells showed that wild type and mutant h-lamp-1 glycoproteins can be detected at the plasma membrane. Wild type h-lamp-1 and reporter hCG-α/G/L^{Y} were efficiently internalized and redistributed to lysosomes, while the mutant glycoproteins lacking tyrosine in the cytoplasmic tail remained at the cell surface. These data suggest lysosomal membrane glycoproteins are transported to lysosomes via the cell surface, and are directed to lysosomes in a process analogous to that used in endocytosis of membrane receptors. [Supported by NIH grant CA48737. M.A.W. is an American Cancer Society Postdoctoral Fellow.]

H 302 **ANALYSIS OF STRUCTURE AND FUNCTION OF THE MR 46000 MANNOSE 6-PHOSPHATE RECEPTOR BY SITE-SPECIFIC MUTAGENESIS**
M. Wendland, R. Pohlmann, A. Hille, A. Waheed and K. von Figura, Georg-August-Universität, Biochemie II, Gosslerstrasse 12d, D-3400 Göttingen, FRG. Mutants of the 46 kDa mannose 6-phosphate receptor (MPR 46) were constructed to elucidate the N-glycoslation pattern, the importance of cysteine residues and amino acids involved in the ligand binding. MPR 46 is a glycoprotein containing 2 complex and 2 high mannose type N-linked oligosaccharides. Deletion of single or multiple glycosylation sites, or inhibition of N-glycosylation by tunicamycin reduce ligand binding in a non-systematic manner. Intramolecular disulfide bonds are essential for proper folding of MPR 46. Four of the six cysteine residues of the luminal domain were substituted by glycine. Each of the four mutants lost the ligand binding, three show an altered glycosylation and two of them are retained in the ER. Chemical modification experiments suggested, that histidine and arginine residues may be present in the ligand binding site. These residues are protected against modification in the presence of ligand. Except for His 131 and Arg 137 all other histidine and arginine residues of the luminal domain can be exchanged without loss of high affinity binding. From these results we expect His 131 and Arg 137 to be located in or close to the ligand binding site.

H 303 INTRACELLULAR LOCATION AND TRANSPORT OF LYSOSOMAL MEMBRANE GLYCOPROTEINS. C. L. Harter, C. L. Howe, M. Hull , B. L. Granger and I. Mellman. Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510.

Lysosomal membrane glycoproteins (lgp) whose cDNAs have been cloned are all characterized by a heavily glycosylated luminal domain, a single transmembrane region and a short cytoplasmic tail (10-11 amino acids). Among the most conserved features is a glycine-tyrosine tandem in the cytoplasmic tail. Since tyrosine residues in the cytoplasmic tails of plasma membrane receptors are often important for coated pit localization, we assumed that this residue might be important for the targeting of newly synthesized lgps to lysosomes, possibly in specifying concentration in clathrin-coated pits in the Golgi apparatus. Using site-directed mutagenesis we have generated different mutations affecting the cytoplasmic tail of rat lgp 120. The mutant proteins have been stably and transiently expressed in CHO and HeLa cells. We have found that mutations changeing just the tyrosine residue appear to affect the normal intracellular targeting of lgp 120 and to result in transport to the cell surface. The posttranslational processing of the tyrosine-mutated lgp however is similar to the wild-type protein. Unexpectedly, pulse-chase labeling of a tail-deleted lgp 120 has shown that this protein seems to be incompletely glycosylated. These results suggest that the cytoplasmic tail of lgp 120 is essential for the correct sorting of lgp 120 to lysosomes as well as for the addition of complex carbohydrates possibly by directing transport from the ER to the Golgi apparatus.The intracellular sorting sites of lgps will be investigated by immunocytochemistry and immunoisolation of vesicles using antibodies directed against different components of the biosynthetic pathway. Supported in part by an EMBO fellowship ALTF 360-1988.

H 305 CARBOXYPEPTIDASE Y - β-GLUCURONIDASE (CPY-GUS) FUSIONS FOR STUDYING THE SORTING OF SOLUBLE PROTEINS TO THE VACUOLE IN S. CEREVISIAE. Martin D. Watson, Mustafa M. Haider, Martin Latterich and Keith H. Leung. Department of Biological Sciences, University of Durham, South Road, Durham, DH1 3LE, UK. We have constructed in-frame fusions between the 5′ end of the PRC1 gene and the *E.coli* GUS gene (*uidA*). The fusion is via an internal BamHI site in PRC1 and a BamHI site upstream from the GUS initiation codon. The CPY domain consists of the signal peptide, the vacuolar sorting domain and 14 amino acids at the N-terminal end of mature CPY. The fusion protein when expressed in yeast is directed to the vacuole where it forms forms an inactive glycoprotein of approximately the same size as native GUS. Site-directed mutagenesis to remove the two glycosylation sites in GUS restores activity. Both glycosylation sites are NXS and in the mutants the serine residues have been changed to alanine. The mutant fusion protein is sorted to the vacuole in wild-type strains, but is secreted in representative class A, B & C *vps* strains. Wild-type colonies expressing CPY-GUS are white on X-gluc agar whereas *vps* strains produce blue colonies. The use of this system to isolate *vps* mutations and to clone the corresponding VPS genes is discussed.

H 306 A GENETIC APPROACH TO THE ISOLATION OF THE YEAST VACUOLAR PROTEIN SORTING RECEPTOR
Carol A. Vater, Luis A. Valls, and Tom H. Stevens, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

In wild-type yeast strains the sorting of newly synthesized procarboxypeptidase Y (proCPY) is highly efficient, with >95% proCPY being targeted correctly to the vacuole. Mutations in the CPY structural gene, PRC1, have been isolated which result in proCPY missorting and secretion (Valls et al., Cell 48, 887-897, 1987). The mutational analysis (Valls, Ph.D. Thesis, 1988) defined a discreet N-terminal vacuolar sorting domain, comprised of amino acids 24-27 of preproCPY (Gln-Arg-Pro-Leu) which is essential for efficient targeting of proCPY to the vacuole.

The availability of yeast strains containing mutations in an essential vacuolar targeting domain of proCPY should allow the isolation of pseudorevertants containing conpensatory mutations in the CPY sorting receptor which result in the restoration of efficient vacuolar targeting. Strains carrying missorting alleles of proCPY, such as *PRC1-ser*$_{27}$ and *PRC1-lys*$_{24}$, secrete 50-80% of their proCPY. These strains have been mutagenized with UV light and screened for the restoration of high levels of intracellular CPY enzyme activity using a plate assay with the CPY substrate N-acetyl-DL-phenylalanine ß-naphthyl ester. Suppression of the mislocalization phenotype was confirmed by quantitative immunoprecipitation of radiolabeled CPY after pulse labeling of cultures with $^{35}SO_4$ and separation into intracellular and extracellular fractions. Mutants exhibiting a suppressed phenotype were subjected to gene substitutions at the *PRC1* locus, using wild-type and missorting alleles. Two of the mutations isolated were found to suppress all *PRC1* missorting alleles tested, including the point mutations *lys*$_{24}$, *ser*$_{27}$, *arg*$_{26}$, and *ser*$_{24}$, as well as a seven amino acid deletion mutation encompassing the sorting domain. One mutation was found to suppress proCPY mislocalization in *lys*$_{24}$ and *ser*$_{27}$ strains only. A yeast strain carrying this allele-specific mutation will be used to clone the suppressor gene.

H 307 A CYTOPLASMIC PROTEIN IN YEAST IS REQUIRED FOR VACUOLAR PROTEIN SORTING, ACIDIFICATION OF THE VACUOLE, AND SEGREGATION OF THE ORGANELLE DURING CELL DIVISION, Christopher K. Raymond and Tom H. Stevens, Institute of Molecular Biology, University of Oregon, Eugene, Oregon, 97403.

We are studying the vacuole of the yeast *Saccharomyces cerevisiae* as a model system for organelle biogenesis. The vacuole is a large, morphologically complex organelle which is actively partitioned between cells during cell division. Newly synthesized vacuolar proteins are transported to the vacuole through the secretory pathway. The lumen of the vacuole is maintained at an acidic pH by a multisubunit H^+-ATPase. Mutants have been isolated which missort newly synthesized vacuolar proteins to the cell surface, and a large number of vacuolar protein sorting (*vps*) genes have been identified. Two of forty *vps* complementation groups examined were also defective for acidification of the vacuole. Extensive characterization of one of these genes, the *VPS3* gene, has been undertaken. The *VPS3* gene encodes a nonabundant, 140 kD cytoplasmic protein whose sequence lacks similarity to other available sequences. While the gene product associates with pelletable cellular material, it is not associated with purified vacuoles. The gene is nonessential as *vps3-Δ1* deletion mutants are viable. Cytoplasmic subunits of the vacuolar H^+-ATPase fail to assemble onto the vacuolar membrane in *vps3-Δ1* cells, consistent with the acidification defect observed in these strains. Fluorescence microscopy revealed that mother to daughter cell vacuolar segregation, mother cell vacuolar morphology, and daughter cell vacuolar biogenesis are all aberrant in *vps3-Δ1* strains. Temperature shift experiments with a conditional *vps3*^{ts} mutant suggest that the *VPS3* gene product participates directly in protein sorting. We currently favor the hypothesis that the Vps3p protein is involved in the assembly of peripheral membrane components onto nascent vacuolar membranes. Additional experiments which examine the role of the *VPS3* gene product in the assembly and maintainance of the vacuole will be presented.

H 308 LOCALIZATION OF THE YEAST VACUOLAR MEMBRANE PROTEIN DPAP B, Chris Roberts and Tom H. Stevens, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

Dipeptidyl aminopeptidase B (DPAP B) is an integral membrane glycoprotein of the yeast vacuole. DPAP B consists of a 29 amino acid NH_2-terminal cytoplasmic tail, a 16 amino acid membrane anchor, and a 795 amino acid lumenal enzymatic domain. To define the targeting signals on this membrane protein, we are characterizing the regions of DPAP B that are necessary and sufficient for its localization to the vacuole. The cytoplasmic and membrane spanning domains of DPAP B are sufficient for its proper targeting, because a hybrid protein consisting of the NH_2-terminal 47 amino acids of DPAP B fused to the cytoplasmic form of invertase is localized to the vacuolar membrane. To determine if the cytoplasmic tail is necessary for targeting, amino acids 2-28 of DPAP B were deleted, leaving only the initiating methionine and an arginine residue on the cytoplasmic side of the membrane. Immunofluorescence experiments showed that the mutant protein resides in the vacuolar membrane, indicating that the cytoplasmic tail of DPAP B is dispensable for vacuolar targeting. Experiments are in progress to assess the roles of the membrane anchor and the lumenal domain in the sorting of this protein.

Another dipeptidyl aminopeptidase, DPAP A, resides in the late Golgi apparatus, and is necessary for proteolytic cleavage of the mating pheromone α-factor. The structure of DPAP A (C. Flanagan and J. Thorner, unpublished) is similar to that of DPAP B in that the two proteins have the same membrane topology, and share a high degree of sequence similarity in their lumenal domains. To determine how two proteins with the same enzymatic activity are localized to distinct compartments within the secretory pathway, the different domains of DPAP A and B have been exchanged. These experiments will also be presented.

H 309 RECONSTITUTION OF PROTEIN TRANSPORT FROM THE GOLGI TO THE VACUOLE OF *S. cerevisiae* IN A CELL-FREE SYSTEM. Karen E. Moore and Tom H. Stevens, Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

To increase our understanding of how eukaryotic cells sort proteins in the Golgi and accurately deliver them to the lysosome, we have developed a cell-free transport system to study the movement of proteins from the Golgi to the lysosome-like vacuole in the unicellular eukaryote, *Saccharomyces cerevisiae*. We have chosen to monitor the delivery of a soluble vacuolar glycoprotein, carboxypeptidase Y (CPY), to the vacuole in this *in vitro* system. CPY serves as an excellent model protein because its movement from the Golgi to the vacuole is accompanied by a proteolytic processing event. Arrival of CPY at the vacuole can be monitored by determining the amount of the vacuolar form present at the end of the reaction using polyacrylamide gel electrophoresis. We have reconstituted this transport reaction using permeabilized yeast cells. Yeast spheroplasts are labeled with ^{35}S-methionine under conditions which optimize the Golgi form of CPY. The plasma membrane of these spheroplasts is permeabilized by subjecting them to an osmotic shock procedure. This technique results in the release of over 90% of a marker cytoplasmic protein and the retention of over 80% of the lumenal contents of the ER and Golgi within the permeabilized cells. Experiments performed using these permeabilized cells have shown us that transport of CPY from the Golgi to the vacuole is temperature dependent, and requires ATP as well as cytosolic proteins. Transport is efficient, with up to 40% of the Golgi form of the enzyme converted to the vacuolar form within a one hour period of incubation. All forms of CPY present at the initial and final stages of the reaction are localized to cellular compartments protected from exogenously added proteases. When detergent is added at the start of the transport reaction maturation of CPY does not occur, indicating that membrane-limited compartments are required for the transport event to occur.

H 310 TARGETING OF YEAST VACUOLAR MEMBRANE PROTEINS, Daniel J. Klionsky and Scott D. Emr, Division of Biology, California Institute of Technology, Pasadena, CA 91125

The yeast vacuole contains a variety of hydrolytic enzymes including both soluble and membrane-associated proteins. In order to analyze the factors affecting delivery of membrane proteins to the vacuole, we examined the biosynthesis of a vacuolar membrane protein, repressible alkaline phosphatase (ALP). Using antiserum specific to ALP, we have been able to demonstrate that ALP is a type II integral membrane protein. Recently, we have undertaken additional studies to identify the vacuolar sorting information in ALP. Fusion of the gene encoding ALP, PHO8, to the SUC2 gene, coding for the normally secreted yeast enzyme invertase, results in the production of a hybrid protein. PHO8-SUC2-encoded hybrid proteins that contain 52 or more N-terminal amino acid residues of ALP are efficiently directed to the vacuole. A deletion of this N-terminal segment from the wild-type ALP protein causes missorting and secretion of the mutated protein. These results indicate that we have identified a vacuolar sorting signal in ALP that is both sufficient and necessary for delivery of this protein to the vacuole. We have also begun to analyze the factors affecting delivery of membrane proteins to the vacuole. Interestingly, some evidence suggests that membrane proteins may be sorted and delivered to the vacuole by a mechanism that is in part different from that used by soluble vacuolar hydrolases. Based on these differences, we are in the process of selecting mutants that have specific defects in the sorting of membrane proteins to the vacuole.

H 311 VACUOLE PROTEIN SORTING AND ORGANELLE ASSEMBLY IN YEAST: GENETIC AND MOLECULAR CHARACTERIZATION OF VPS16, Bruce F. Horazdovsky and Scott D. Emr, Division of Biology, California Institute of Technology, Pasadena, CA 91125. The S. cerevisiae vacuolar protein sorting mutant, vps16, exhibits pleiotropic defects in vacuolar protein targeting and vacuolar morphology. The gene product affected by this mutation may represent a basic component of the vacuolar protein targeting machinery or may be involved in the formation of key structural intermediates of the vacuole. To better understand the process of vacuolar protein localization and vacuolar biogenesis, the vps16 mutant was used to clone the wild-type allele of the affected gene. Taking advantage of the ts growth phenotype of one of the vps16 mutant alleles, five complementing clones were isolated from a yeast genomic library, all of which shared related sequences. The complementing activity was further defined and found to be contained on a 2.2 Kb genomic DNA fragment. Integrative mapping studies have been used to demonstrate that this minimum complementing fragment is tightly linked to the vps16 mutant locus. Gene disruption experiments have shown that VPS16 is not essential, but rather that the null mutation leads to a ts growth defect. Preliminary DNA sequence analysis has identified an open reading frame within the VPS16 complementing fragment capable of encoding a protein of at least 58,000 MW. The deduced amino acid sequence of this open reading frame shows no significant sequence similarities with other known proteins. TrpE-Vps16 fusion proteins have been generated and are being used for the production of polyclonal antisera. These antibodies will be used to study the biosynthesis and the subcellular localization of the VPS16 gene product.

H 312 Sorting mechanisms of barley lectin to the vacuoles of plant cells, Sebastian Y. Bednarek, Thea A. Wilikins and Natasha V. Raikhel, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312

Plant vacuolar proteins like other secretory proteins enter and are processed through the ER/Golgi/trans-Golgi network. Sorting of yeast and mammalian secretory proteins has been found to be directed by selective retention or targeting information contained within the structure of the protein. Plant, yeast and mammalian proteins lacking sorting information appear to follow a default pathway through the secretory system. To understand the mechanisms of protein targeting to the plant vacuole we are attempting to define and characterize the sorting signal(s) of the vacuolar protein Barley lectin (BL). BL is synthesized as a preproprotein with a transiently associated glycosylated carboxyl-terminal propeptide which is removed prior to or concomitant with deposition of the mature protein in the vacuole. Expression of a gene for BL in transformed tobacco plants and suspension-cultured cells (NT cells) results in the correct processing and accumulation of active mature barley lectin in the vacuole. Similarly, active mature BL is localized within the vacuoles of plants which express a mutated gene of BL where the glcosylation site of the propeptide has been modified to prevent attachment of the high-mannose glycan. Therefore, the glycan domain of the carboxyl-terminal propeptide does not function as a vacuolar sorting determinant. Pulse chase experiments in transgenic tobacco protoplasts however show that the glycan may modulate the rate of processing of the BL proprotein through the Golgi complex. Preliminary results show that expression of a mutant gene of BL lacking the carboxyl-terminal propeptide does not result in accumulation of BL in transgenic tobacco vacuoles. Therefore the carboxyl-terminal propeptide may function as a vacuolar sorting signal. To test this hypothesis we will determine if the carboxyl-terminal propeptide is sufficient to redirect a cytosolic reporter protein to the vacuole. We have prepared chimeric genes containing the carboxyl-terminal propeptide of BL fused with a cytosolic reporter protein and have transformed the constructs into both plants and NT cells. Analysis of the protein fusions are in progress and will be facilitated by use of the NT cells because of their fast generation times and suitability for organelle fractionation.

H 313 THE CELL-CYCLE CONTROL PROTEIN KINASE cdc2 INHIBITS ENDOCYTIC VESICLE FUSION IN VITRO, Jean Gruenberg, Teppo Tuomikoski, Marie-Anne Felix and Marcel Dorée. Cell Biology Programe, European Molecular Biology Laboratory, D-6900 Heidelberg, F.R.G.

In animal cells, several steps of membrane traffic are arrested during mitosis. To investigate the mechanism of this regulation, we have used a cell-free assay of endocytic vesicle fusion. Two cytosol-free endosomal fractions were prepared from BHK cells after fluid phase endocytosis for 5 min at 37°C of avidin (Av) and biotinylated HRP (bHRP) respectively. In the assay, these fractions were mixed with concentrated Xenopus cytosol, incubated at 37°C and the fusion was quantitated by the formation of Av-bHRP complex using an ELISA. Fusion was reduced in the presence of mitotic cytosol, when compared to interphase cytosol. More important, fusion inhibition could be reconstituted by complementing interphase cytosol with a purified preparation of the the cell-cycle control protein kinase cdc2. Inhibition was prevented after cdc2 depletion by affinity-binding to the fission yeast protein p13suc1 and restored with the affinity-purified cdc2 kinase. Maximal inhibition (>90%) occured when the cdc-2 activity present in the assay, measured by H1 histone phosphorylation, was in the same range as in mitotic extracts. These findings suggest that endocytic vesicle fusion, and possibly other fusion events in membrane traffic, are inhibited during mitotis and that this inhibition is mediated by the cell-cycle control protein kinase cdc2.

H 314 PARATHYROID HORMONE DECREASES DENSITY OF Na^+-phosphate (P_i) SYMPORTERS IN BRUSH BORDER MEMBRANE (BBM) OF RENAL PROXIMAL TUBULES, T.P. Dousa, A. Hoppe, J.-T. Lin, M. Onsgard, and F.G. Knox, Mayo Clinic and Mayo Medical School, Rochester, MN 55905. We tested the hypothesis that parathyroid hormone (PTH) decreases the capacity for Na^+-P_i symport across renal BBM by a selective internalization of Na^+-P_i symporters. We employed the Na^+-dependent P_i-suppressible binding of [^{14}C]-phosphonoformic acid (Na^+-^{14}C-PFA) on BBM vesicles (BBMV) for detection of density of Na^+-P_i symporters; ^{3}H-phlorizin binding was employed for detection of the density of Na^+-D-glucose symporters in the same membrane. BBMV prepared from renal cortex of control rats and rats infused with PTH were analyzed for ligand binding and for transport activity. In BBMV from PTH-infused rats, the B_{max} for Na^+-^{14}C-PFA binding to BBMV was markedly decreased (-38%) in parallel with a decrease in rate of the Na^+-gradient-dependent $^{32}P_i$ uptake by BBMV (-43%). In contrast, in the same BBMV preparations the B_{max} for ^{3}H-phlorizin binding and the rate of Na^+-gradient-dependent uptake of ^{3}H-D-glucose were not different between control and PTH-treated rats. Also, we found no differences in activities of alkaline phosphatase and other intrinsic BBM enzymes, or in content of immunoreactive actin between BBMV from control and from PTH-treated rats. The results indicate that a decrease in the number of Na^+-P_i symporters in the luminal surface of BBM (with concomitant decrease in Na^+-P_i symport) caused by PTH is the specific event, compared to other intrinsic components of BBM. We suggest that PTH elicits internalization (endocytosis) of Na^+-P_i symporters from BBM surface to subapical cytoplasm.

H 315 **Role of Palmitylation of the Human Transferrin Receptor**

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The human and murine transferrin receptors are post-translationally modified by the addition of covalently bound fatty acid. The major site of acylation of the human transferrin receptor has been identified as cysteine-62. However, the role of acylation at cysteine-62 is unclear because this residue is not conserved in the murine transferrin receptor. Further studies are therefore required in order to identify the sites of acylation and to understand the functional role of transferrin receptor acylation. We performed site-directed mutagenesis of the human transferrin receptor cDNA to replace the potential sites of acylation with serine and alanine residues. Wild-type and mutated human transferrin receptors were expressed in cultured CHO cells that lack functional transferrin receptors (clone TF$^-$). The properties of the transferrin receptors expressed in CHO cells were investigated by measuring the rates of receptor endocytosis and recycling. It was found that the rate of recycling of the wild-type and mutant receptors were not significantly different. However, the rate of endocytosis of the mutant receptors was markedly greater than that observed for the wild-type transferrin receptor. These results indicate that the covalent modification of the transferrin receptor with palmitate may play a role in the regulation of receptor cycling.

H 316 MEETING OF THE APICAL AND BASOLATERAL ENDOCYTIC PATHWAYS OF THE MDCK CELL, Robert G. Parton, Kai Simons and Gareth Griffiths, European Molecular Biology Laboratory, Heidelberg, W. Germany.

Electron microscopy was used to characterise the endocytic pathways in polarised monolayers of MDCK cells grown on filters. The apical and basolateral pathways were shown to have distinct sets of tubulovesicular early endosomes which filled with fluid-phase markers approximately 10 min after the onset of internalisation and were located in a peripheral position in the cell. Apical and basolateral markers met after 15 min at 37°C in cation-independent mannose-6-phosphate receptor (MPR)-enriched late endosomes (prelysosomes) predominantly located in the Golgi region of the cell but also found basal to the nucleus. After longer incubation times, ligands from both surfaces accumulated in MPR-negative lysosomes located almost exclusively in the supranuclear portion of the cell. Basolaterally-internalised fluid-phase markers also accumulated after a lag period in tubular structures located under the apical surface. Such structures are apparently distinct from apical early endosomes and may be involved in transcytosis.

Pretreatment of cells with nocodazole to depolymerise the microtubules was shown to dramatically reduce meeting of apical and basolateral endocytic markers in MPR-positive late endosomes whereas intracellular accumulation of fluid-phase markers was virtually unaffected. The markers were restricted to the periphery of the cell and were principally observed in two classes of structures; tubulovesicular structures, presumably early endosomes, and spherical structures, morphologically similar to the putative carrier vesicles described in non-polarised cells (Gruenberg et al., 1989, J. Cell Biol. 108: 1301-1316). The latter may be involved in microtubule-dependent transport between the two sets of early endosomes and the late endosomes of the MDCK cell.

H 317 INTRACELLULAR TRANSPORT OF CYTOCHROME C AND LYSOZYME IN RAT KIDNEY

Helge Tolleshaug[1], Jan Hysing[2] and Norman P. Curthoys[3]

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The intracellular transport in the kidneys of the two small, basic proteins lysozyme and cytochrome c were studied and shown to follow distinct pathways. The two proteins were labeled with a residualizing label (^{125}I- or ^{131}I-tyramine-cellobiose (TC)) in order to follow the degradation products as well as the intact proteins, and kidney tissue was fractionated in sucrose density gradients. Both proteins are cleared from the blood with very similar kinetics; they are filtered in the glomeruli and taken up from the ultrafiltrate by receptors in the brush border. Immediately after injection into rats, both proteins showed very similar intracellular distributions. They were found in light vesicles of the same density as the plasma membrane. After 5 min, their distribution profiles started to diverge. ^{125}I-TC-cytochrome c and its degradation products were found mainly in the lysosomal region of the gradient; ^{131}I-TC-lysozyme was transferred into the lysosomes at a much slower rate. Our observations support the idea that lysozyme and cytochrome c are absorbed from the ultrafiltrate by the same receptor, but they are sorted into different vesicles after endocytosis and transported by different pathways.

H 318 A UNIQUE SUBSET OF CYTOPLASMIC PROTEINS IS ASSOCIATED WITH ENDOSOMES.

Peter van der Sluijs, Bruno Goud¶, and Ira Mellman, Dept. of Cell Biology, Yale University School of Medicine, New Haven, CT 06510 and ¶Dept. of Immunology, Institut Pasteur, Paris, France. Endocytic uptake of extracellular ligands is followed by a coordinated series of selective and specific intracellular sorting and fusion events. The mechanisms whereby the cell exerts control over these events depend very likely on as yet unidentified cytoplasmic and membrane associated molecules. We therefore looked at cytoplasmic proteins which associate specifically with endosomes. Highly purified endosomes from CHO cells were isolated by discontinuous sucrose centrifugation and free flow electrophoresis. Upon incubation with homologous ^{35}S-labeled cytosol, a specific subset of proteins with molecular weights of 19, 21, 46, 49, 85, and 126 kD was bound to endosomes, but not to an irrelevant membrane fraction as analyzed by PAGE and fluorography. Currently we are characterizing these proteins and the factors required for binding. In addition we have started to investigate whether isolated endosomes contain GTP binding proteins as these proteins are required for protein transport between compartments of the secretory pathway and also seem to be involved in endosome fusion (Diaz, et al. Nature 339: 396, 1989). Endosomal proteins were resolved by PAGE and transferred to nitrocellulose membranes. Western blotting with a panel of anti rab antibodies showed that the purified endosomes contained a YPT1p/SEC4p related rab protein. Probing these blots with α-[^{32}P] GTP showed that the purified endosomes contained two GTP binding proteins with molecular weights of 22.5-25 kD which remained associated with the organelles upon mild trypsin treatment. We are now investigating the nucleotide specificity and the GTPase activity of these proteins, and whether these proteins are restricted to a specific class of endosomes.

H 319 THE MANNOSE 6-PHOSPHATE RECEPTOR CYTOPLASMIC DOMAIN IS NOT SUFFICIENT TO ALTER THE CELLULAR DISTRIBUTION OF A CHIMERIC EGF RECEPTOR, S.M. Dintzis and S.R. Pfeffer, Department of Biochemistry, Stanford University School of Medicine, Stanford,CA 94306-5307

Cytoplasmic domains play an important role in the collection of cell surface receptors into clathrin-coated pits and their subsequent endocytosis. Unlike most receptors, 300kD mannose 6-phosphate receptors (MPRs) are localized primarily in the trans Golgi network (TGN) and endosomes (~95%), and they cycle constitutively between these compartments. Yet, when present at the cell surface, MPRs are internalized together with other cell surface receptors in clathrin-coated vesicles. We constructed a chimeric receptor to test whether the MPR cytoplasmic domain contained sufficient information to direct a cell surface receptor into both of these transport pathways. Molecular cloning techniques were used to precisely join the human EGF receptor extracellular and transmembrane domains with the cytoplasmic domain of the bovine MPR. The expressed chimeric protein formed a stable structure with a half life of ~20 hr, bound EGF with high affinity (Kd~0.9X10^{-9}M), and was efficiently endocytosed and recycled back to the cell surface. If the cytoplasmic domain alone is responsible for sorting native MPRs, chimeric receptors might have been expected to be located primarily in the TGN and in endosomes at steady state. To our surprise, while endogenous MPRs were essentially all intracellular, >85% of the chimeric receptors were located at the cell surface. Chimeric receptors were not trapped at the surface because they internalized anti-EGF receptor Fab fragments in the presence or absence of EGF. These experiments demonstrate that the MPR cytoplasmic domain is not sufficient to alter the steady state distribution of the EGF receptor, and suggest a role for MPR extracellular and transmembrane domains in the cellular routing of this receptor. We propose that these MPR domains contain an "endosome retention" signal that facilitates the movement of this receptor from early to late endosomes, thereby facilitating its return to the TGN.

H 320 A COMPARISON OF THE INTRACELLULAR PATHWAYS OF ENDOCYTOSIS IN ENDOTHELIAL, PARENCHYMAL AND KUPFFER CELLS OF LIVER.

Grete M. Kindberg, Helge Tolleshaug* and Trond Berg, Institute for Nutrition Research, University of Oslo, Norway and *NYCOMED, Oslo, Norway.

Mannose-terminated glycoproteins, asialoorosomucoid and air-filled albumin microspheres (Albunex®) are selectively taken up by endocytosis in rat liver endothelial, parenchymal and Kupffer cells, respectively. The intracellular transport and degradation of endocytosed material were studied by means of subcellular fractionation in sucrose and Nycodenz gradients following intravenous injection of the ligand. By using ligands labelled with ^{125}I-tyramine-cellobiose the subcellular distribution of labelled degradation products could be studied, since they are trapped at the site of formation (e.g. lysosomes).

During the first minutes after internalization in endothelial cells, the ligand is associated with two types of endosomes and then rapidly transferred to lysosomes. The degradation seemed to take place sequentially in two subclasses of lysosomes ("transfer" and "accumulation" lysosomes) that could be separated in Nycodenz gradients. In sucrose gradients, the two types of lysosomes could be separated after allowing the cells to accumulate invertase. The lysosomes may be connected in such a way that the labelled degradation product is recycled between them.

In parenchymal cells, asialoorosomucoid is located in a relatively slow sedimenting vesicle during the first minute after internalization and subsequently in denser endosomes. Degradation products were detected in the gradient after 15 minutes and accumulated in denser regions of the gradients. The rate-limiting step of proteolysis in parenchymal cells is probably the transport from endosomes to lysosomes.

In Kupffer cells, the major portion of albumin microspheres is found as undegraded material in very dense endosomes up to 3 hours after injection. After 24 hours, most of the ligand is degraded in a lysosome distributed in lighter regions than the endosomes in both Nycodenz and sucrose gradients.

H 321 CELL SURFACE TRANSPORT, OLIGOMERIZATION AND ENDOCYTOSIS OF CHIMERIC TYPE II GLYCOPROTEINS,Amitabha Kundu,M. Abdul Jabbar and Debi P. Nayak,Department of Microbiology and Immunology,UCLA School of Medicine,Los Angeles,CA 90024. Chimeric genes were constructed between gene segments encoding type II glycoproteins [influenza virus neuraminidase (NA) and human transferrin receptor (TR)] and expressed in mammalian cells to investigate the role of cytoplasmic and anchor domains in intracellular transport, processing, oligomerization and endocytosis. The chimeric proteins in which domains were precisely exchanged were productively targeted to the cell surface. We also analyzed the subunit composition of the wt and the chimeric glycoproteins. The influenza NA and TR were present as a homotetramer and a homodimer, respectively. However, NATRΔ90, in which the cytoplasmic and anchor domains of NA were appended to the ectodomain of TR, was present as tetramer, dimer, as well as monomer. TRNAΔ35, in which the cytoplasmic and anchor domains of TR were joined to the ectodomain of NA, existed predominantly as a dimer, suggesting that the cytoplasmic and anchor domains of type II glycoproteins might affect the subunit assembly of the ectodomains. We also analyzed the role of cytoplasmic domain in endocytic uptake. TR but not NA was capable of being sequestered into coated pits and internalized. The chimera NATRΔ90 failed to undergo endocytosis whereas TRNAΔ35 was internalized, albeit inefficiently. These studies reinforced the importance of cytoplasmic domain in receptor recycling pathway and also showed that the NH_2- cytoplasmic domain of a type II membrane protein alone possesses sufficient and necessary information to direct a heterologous extracellular domain into the endocytic vesicles.

H 322 **TRAFFICKING OF HERPES SIMPLEX VIRUS GLYCOPROTEIN B-1 IN TRANSFECTED COS CELLS,** J.D. Fetherston and R.J. Courtney, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, LA 71130.

Glycoprotein B-1 (gB-1) of herpes simplex virus is required for the production of infectious viral particles. A variety of experimental evidence suggests that gB-1 is involved in the entry of the virus into the cell, possibly by mediating fusion of the viral envelope to the cellular membrane. However, the regions of the protein required for this fusion event have not been identified. We have examined the synthesis and processing of gB-1 in Cos-1 cells using a transient expression system. Cos-1 cells transfected with a wild-type gB-1 gene contain large cytoplasmic vacuoles which react with monoclonal antibodies to gB-1. Treatment of the cells with either monensin or chloroquine prevents the appearance of these vacuoles. Furthermore, monoclonal antibodies directed against gB are internalized by cells expressing gB-1, and localize to the cytoplasmic vacuoles. These results suggest that the large vacuoles are derived from fusion of endocytic vesicles containing gB-1. Cos cells transfected with linker insertion mutants that express carboxy-terminal truncated forms of gB-1 (W. Cai, S. Person, C. DebRoy, and B. Gu, 1988 J.Mol. Biol. **201**:575-588), do not form the vacuoles.

H 323 **Role of Tyrosine^{20} in the Cytoplasmic Domain of the Transferrin Receptor.**
Núria Gironès, Elvira Alvarez and Roger J. Davis, Department of Biochemistry, University of Massachusetts Medical School, 55 Lake Ave N., Worcester, MA 01655.

The rate of receptor- mediated endocytosis of diferric ^{125}I-transferrin by CHO cells expressing human transferrin receptors was compared with the rate measured for cells expressing hamster transferrin receptors. The rate of endocytosis of the human transferrin receptor was significantly faster than the hamster receptor. In order to examine the molecular basis for the difference between the observed rates of endocytosis, a hamster transferrin receptor cDNA clone was isolated. The predicted primary sequence of the cytoplasmic domain of the hamster transferrin receptor is identical to the human receptor except at residue 20 where a tyrosine in the human sequence is replaced with cysteine. To test the hypothesis that this structural change in the receptor is related to the difference in the rate of internalization we used site-directed mutagenesis to examine the effect of the replacement of tyrosine^{20} with a cysteine residue in the human transferrin receptor. It was observed that the substitution of tyrosine^{20} with cysteine caused a 62% inhibition of the rate of iron accumulation by cells incubated with [^{59}Fe]diferric transferrin. No significant difference between the rate of internalization of the mutant (cysteine^{20}) human receptor and the hamster receptor was observed. Thus, the substitution of tyrosine^{20} with a cysteine residue can account for the difference between the rate of endocytosis of the human and hamster transferrin receptors.

H 324 RECYCLING OF PLASMA MEMBRANE PROTEINS TO THE GOLGI APPARATUS IN ENDOCRINE CELLS. Samuel A. Green and Regis B. Kelly. Department of Biochemistry, University of California Medical School, San Francisco, California 94143.

There has long been morphological evidence that secretory cells recycle membrane components from the plasma membrane to the Golgi apparatus following exocytosis. Recently it has been shown that fibroblast and lymphoma cell lines can selectively recycle the mannose-6-phosphate receptor from the cell surface to the Golgi apparatus (Duncan, J. R. and S. Kornfeld, J Cell Biol. 106:617). To compare this membrane pathway in regulated secretory cells and fibroblastic cells, we have selected a variant of the neuroendocrine PC12 cell line for its resistance to ricin. The variant cells are deficient in terminal galactose addition to their glycoproteins, making them useful for the study of glycoprotein traffic from the plasma membrane to the trans Golgi network, the site of sialic acid addition, as described by Duncan and Kornfeld. We have found that PC12 cells recycle a larger number of cell surface proteins to the Golgi apparatus than do CHO fibroblasts. The recycling is blocked at 18°C. In addition, we have found that the synaptic vesicle membrane protein synaptophysin (p38) follows this route at least twice as efficiently as total cell surface proteins. We are currently comparing the recycling of synaptophysin with specific markers for other membrane compartments including the plasma membrane and dense core secretory vesicles.

H 325 SELECTIVE UPTAKE OF YOLK PROTEINS BY DROSOPHILA MELANOGASTER OOCYTES, Alberto Martinez, Katrin Lineruth-Sitbon and Mary Bownes, Department of Molecular Biology, University of Edinburgh, King's Buldings, Mayfield Rd., Edinburgh, EH9 3JR, UK. Vitellogenesis in insects involves the selective uptake of Yolk Proteins (YPs) by the oocyte. The function of the YPs is to provide nutrients during embryo development. Recently, the YPs have been postulated to bind apolar conjugates of ecdysteroids, which may be released during embryogenesis prior to cuticle secretion. One site of YP synthesis is the follicle cells surrounding the oocyte, the other site is the fat body of adult female flies from which the proteins are secreted into the hemolymph. The YPs accumulate in the oocyte via receptor mediated endocytosis. The aim of our study is to determine the protein domains of the YPs involved in the interaction with the receptor. To this end a dual strategy was devised. One approach consisted of altering the structure of the YP genes by *in vitro* mutagenesis and analysing the effects on secretion and uptake *in vivo*. The second approach involved assaying the YPs of other Dipteran species for their uptake into *D. melanogaster* oocytes with a view to cloning and sequencing the genes of evolutionary distant YPs with conserved uptake function. The assay consisted of hemolymph transplants between the different species. The results of experiments using these two approaches are presented.

H 326 A MEMBRANE-PROXIMAL TYROSINE RESIDUE IN THE CYTOPLASMIC TAIL IS NOT REQUIRED FOR EFFICIENT ENDOCYTOSIS OF A MURINE Fc RECEPTOR. Heini M. Miettinen, Walter Hunziker, John K. Rose and Ira Mellman. Departments of Pathology and Cell Biology, Yale University School of Medicine, New Haven, CT 06510.

We have previously shown that two isoforms of the murine IgG1/IgG2b Fc receptor, FcRII-B1 and -B2, exhibit markedly different abilities to localize in coated pits. FcRII-B1 and -B2 are identical, except for a 47 amino acid insert in the cytoplasmic tail of B1. B1 does not localize in coated pits and is poorly internalized. A tail-minus mutant, with only one amino acid in the cytoplasmic tail is similar to B1 with respect to coated pit localization and internalization efficiency. Taken together these results suggest that the cytoplasmic tail of B2 contains a domain that is necessary for coated pit localization and subsequent endocytosis. Cytoplasmic tails of many receptors have been shown to contain a tyrosine residue that is critical for coated pit localization. The cytoplasmic tail of B2 contains two tyrosines (positions 26 and 43) and that of B1 contains three tyrosines (positions 28, 54 and 73). To investigate whether the tyrosine in position 26 of B2 is critical for endocytosis, it was mutated to an alanine. Conversely, a glycine in position 26 of B1 was mutated to a tyrosine to examine whether this would allow endocytosis of this receptor. Preliminary results using immunofluorescence of transiently-expressing COS cells suggest that the Tyr26 in B2 is not required for endocytosis and that the acquired Tyr26 in B1 is not sufficient to allow endocytosis. Carboxy-terminus deletion mutants of B2 with 18 (CT18) or 31 (CT31) amino acids remaining in the cytoplasmic tail were also internalized relatively efficiently, with CT31 being somewhat faster than CT18. This indicates that neither the membrane-proximal tyrosine nor the domain surrounding it is necessary for internalization. We are now in the process of making stably transfected CHO cell lines to examine by EM what effect, if any, these mutations have on coated pit localization.

H 327 MODULATION OF THE ENDOCYTIC UPTAKE OF RICIN AND FLUID PHASE MARKERS WITHOUT ALTERATION IN TRANSFERRIN ENDOCYTOSIS. Kirsten Sandvig[1] and Bo van Deurs[2], [1]Institute for Cancer Research at the Norwegian Radium Hospital, Oslo 3, Norway; [2]Structural Cell Biology Unit, Department of Anatomy, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark. In Vero cells cytochalasin D was found to reduce the endocytosis of ricin and the fluid phase markers [^{14}C]sucrose and lucifer yellow without reducing the uptake of transferrin. The number of coated pits at the plasma membrane was not affected by the treatment. Cytochalasin D also reduced the endocytosis of ricin in acidified cells where uptake of transferrin from coated pits was blocked by low cytosolic pH. In A431 cells EGF and TPA increased the endocytic uptake of ricin both under normal culture conditions and when the coated pit/coated vesicle pathway was blocked by acidification of the cytosol. In contrast, EGF and TPA had no stimulatory effect on the uptake of transferrin at normal cytoplasmic pH, and EGF and TPA did not abolish the ability of low cytoplasmic pH to inhibit endocytic uptake of transferrin. The results indicate that cytochalasin D selectively inhibits endocytic uptake from non-clathrin coated areas of the cell membrane whereas EGF and TPA stimulate it. The data therefore support the view that there are different endocytic mechanisms, and the results suggest that at least in some cell types one can modulate non-clathrin coated endocytosis.

H 328 DIFFERENTIAL BLOCKS IN TRANSPORT FROM THE ER TO THE GOLGI OF YEAST *IN VITRO*: UNIQUE REQUIREMENTS FOR VESICLE FORMATION, TARGETING AND FUSION Michael Rexach and Randy Schekman, Division of Biochemistry and Molecular Biology, University of California, Berkeley CA 94720

Intercompartmental protein transport is mediated by small vesicle carriers which bud from a donor compartment and fuse with their specific target compartment. To understand the mechanics of this process we developed an *in vitro* system which reconstitutes transport from the ER to the Golgi compartment in gently-lysed yeast spheroplasts. We have recently developed a subassay which separates the requirements for vesicular exit from those of vesicular targeting and fusion. The assay exploits a peculiarity of gently-lysed yeasts spheroplasts, namely, that the Golgi compartment and ER to Golgi vesicles are released from the broken cells during assay conditions and thus sediment slowly,while the donor ER remains associated with the broken cells and sediments rapidly.Vesicle formation is quantified by the release of core glycosylated α factor precursor from the broken cells. The intermediate vesicles which are released can either be attached but not fused with the Golgi compartment (intermediates in fusion) or unattached (intemediates in targeting). The unattached vesicle intermediates can be physically separated from the Golgi compartment by sedimentation in density gradients.

We have identified seven blocks in the *in vitro* transport reaction: sec12, sec23, sec18, ypt1,depletion of free Ca^{++}, NEM and GTPγS. All these blocks accumulate only the core glycosylated form of the α factor precursor.The *in vitro* blocks generated by sec12, sec18 and sec23 are thermolabile and the block generated by depletion of Ca^{++} is fully reversible. We have characterized their single and/or multiple sites of action at vesicle formation, targeting or fusion. We find that vesicular exit requires cytosol, ATP, functional Sec12 and Sec23 proteins and is sensitive to GTPγS and NEM.Vesicular targeting requires functional Sec18 and Ypt1 proteins. Finally, vesicular fusion requires the presence of free Ca^{++}, ATP, cytosolic factors and is inhibited by NEM. In summary, these differential blocks allow us to study the individual proteins and requirements underlying the mechanisms of vesicle formation, targeting and fusion. Moreover, they facilitate the purification of vesicles in order to study their structural components.

H 329 THE Sec23 PROTEIN FUNCTIONS AS PART OF A LARGE, PERIPHERAL MEMBRANE COMPLEX IN THE TRANSPORT OF SECRETORY PROTEINS FROM THE ER TO THE GOLGI CISTERNAE
Linda Hicke and Randy Schekman, Division of Biochemistry and Molecular Biology, University of California, Berkeley CA 94720

The protein encoded by the *SEC23* gene is required for intracellular protein transport from the endoplasmic reticulum to the Golgi apparatus. Temperature-sensitive mutations in *SEC23* cause accumulation of secretory proteins in the ER and proliferation of ER membrane at the restrictive temperature. In addition, in an in vitro ER to Golgi transport assay transport in *sec23* lysates is temperature-sensitive. We have developed this assay for purification of the Sec23 protein. Specifically, transport at the nonpermissive temperature (30°C) in *sec23* membranes supplemented with *sec23* cytosol is reduced five-fold relative to transport at the permissive temperature (15°C). Supplementation with cytosol containing the wildtype *SEC23* gene product restores transport at 30°C. Addition of partially purified fractions of wildtype Sec23 protein also restores transport in mutant lysates at 30°C. Sec23p activity was purified using ion-exchange chromatography and gel filtration. The activity copurifies with two polypeptides of 85 kD and 105 kD, and behaves on gel filtration columns as a protein of 400 kD.

The predicted amino acid sequence of *SEC23* encodes a protein of 84.5 kD and Sec23p antisera recognize a protein that migrates as 85 kD on SDS-polyacrylamide gels. Overproduction of this protein allows purification of a momomer form of Sec23p that is active in ER to Golgi transport in vitro. Differential centrifugation of yeast lysates under various buffer conditions indicates that the Sec23 protein is peripherally associated on the cytosolic surface of intracellular membrane. We believe that the 105 kD protein and Sec23p act in ER to Golgi transport as a multiprotein complex loosely associated with a membrane surface.

H 330 PGP63, A POSSIBLE MODULATOR OF THE YEAST SECRETORY PATHWAY, Timothy A. Fritz, Piotr Kulesza, David M. Bedwell, and Richard B. Marchase,
Departments of Cell Biology & Anatomy and Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294.

A cytosolic phosphoglycoprotein containing phosphodiester-linked Glc with an apparent molecular mass of approximately 63 kDa (pgp63) has been implicated in the regulated secretory processes of Paramecia and PC12 cells. In addition, antibodies against the Paramecium protein cross-react with pgp63 in rat liver, a constitutively secreting organ. Incubation of wild-type (strain X2180-1A) yeast homogenate with the ^{35}S-labeled β-phosphorothioate analogue of UDP-Glc results in the labeling of a single protein of approximately the same molecular mass. This phosphoglycoprotein shows similar pI and chemical characteristics to Paramecia, PC12, and rat liver pgp63. Yeast secretory mutants were screened for the pgp63$^+$ phenotype. In initial studies, three mutants (sec 3-2, sec 4-2, and sec 8-6) associated with a late secretory event displayed a pgp63$^-$ phenotype in homogenate assays. Analyses of highly back-crossed sec 3, 4, and 8 strains (from P. Novick), however, showed a pgp63$^+$ phenotype. It was subsequently determined that each of the initial mutants was derived from NF1R, a spontaneous Gal$^+$ hypersecretory revertant of X2180-1A. The involvement of pgp63 in the regulated secretory systems mentioned above, together with the results in yeast, suggest a possible gating role for pgp63 in the constitutive yeast secretory pathway.

H 331 H-*ras* ONCOGENE INDUCES PANCREATIC β-CELL DYSFUNCTION AND DEGENERATION IN MALE INSULIN-*RAS* TRANSGENIC MICE. Shimon Efrat and Douglas Hanahan*, Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, and *Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143.

Transgenic mice expressing an insulin-promoted H-*ras* hybrid gene in pancreatic β cells develop β-cell degeneration and diabetes. The disease is manifested in male mice by hyperglycemia, glycosuria, and reduced plasma insulin levels, which appear around 5 months of age and lead to premature death. Histological analyses reveal large holes within the islets of Langerhans and a reduced number of β cells. The destruction of the islets is not associated with an obvious inflammatory activity. Ultrastructural analysis shows extensive engorgement in the endoplasmic reticulum of the residual β cells from diabetic males. The data suggests that β-cell degeneration is preceded by impaired insulin secretion. The insulin-ras females do not manifest any of the physiological abnormalities observed in males and show only minor histological and ultrastructural changes, even at much older ages. The H-*ras* oncoprotein is expressed at comparable levels in the β cells of males and females, where it does not in either case elicit cell proliferation. The differential sensitivity to H-*ras* suggests that males and females differ in their regulation of β-cell function, perhaps to accommodate their distinct metabolic requirements, such as during pregnancy in females.

H 332 RAS-LIKE GENE FAMILY OF THE FISSION YEAST SCHIZOSACCHAROMYCES POMBE.

Erica H. Fawell, Sally D. Hook and John I. Armstrong, Membrane Molecular Biology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX England.

Recently two budding yeast genes required for different steps in membrane traffic, SEC4 and YPT1, have been shown to be members of a family of GTP-binding proteins whose prototype is the mammalian *ras* gene product. The *ras* proteins bind guanine nucleotides, exhibit GTPase activity and thus resemble the G-proteins which are involved in mediating signal transduction. It has been proposed that a *ras*-related family of G-proteins is involved in eukaryotic membrane traffic, with different members targeting successive stages in vesicular transport.

We are cloning members of this family from the fission yeast Schizosaccharomyces pombe using a mixed oligonucleotide to the highly conserved GTP-binding region in *ras* proteins. We have previously reported the cloning and sequence analysis of the S. pombe YPT1 homolog (1). Several genomic clones have also been isolated with the *ras* oligo and appear to have significant homology to YPT1. Using a variety of genetic and biochemical methods we are investigating the role of these potential membrane traffic genes. Gene inactivation/replacement studies are in progress to investigate the phenotype of null alleles of these genes. Using an approach first used with the budding yeast YPT1 gene, a mutation is introduced at a site whose sequence is conserved in most of the *ras* family. The corresponding mutation in YPT1 and SEC4 cause dominant arrests in membrane traffic. By introducing this mutation into our genes, under the control of an inducible S. pombe promoter, the effects on growth and membrane traffic are being studied.

1. Fawell, E., Hook, S. and Armstrong, J. (1989) NAR **17**, 4373.4

H 333 GENES WHICH CAN MAKE *S. CEREVISIAE* INDEPENDENT OF Ypt1p, A 23 kD GTP-BINDING PROTEIN INVOLVED IN SECRETION, C. Dascher, R. Ossig, H.D. Schmitt, and D. Gallwitz, Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, D-3400 Göttingen, FRG.

The *YPT1* gene in *S. cerevisiae* codes for a 23 kD GTP-binding protein essential for cell growth. Conditional-lethal *ypt1* mutants at the restrictive temperature display several phenotypic alterations, the most prominent of which is a defect in protein secretion. We have identified a mutant gene (*SLY1*) which is able to suppress the deletion of the *YPT1* gene at temperatures below 33ºC. The DNA sequence of two identified *SLY1* mutant and the *SLY1* wild type alleles showed that particular point mutations within a 1998 bp open reading frame lead to mutant proteins able to complement the Ypt1p deficiency. In addition, three multicopy suppressors, *SLY2*, *SLY12* and *SLY41* were identified. None of the *SLY* genes codes for a GTP-binding protein, instead *SLY2*, *SLY12* and *SYL41* encode potential membrane-bound proteins. Evidence has been obtained to suggest that the *SLY* genes might be involved at particular stages of intracellular protein traffic:

- Besides the *YPT1* deficiency, *SLY1-20*, *SLY2* and *SLY12* can also partially suppress other secretion defects in yeast (*sec21* and *22* - but not *sec4, 7, 12, 13, 16, 18, 20, and 23*).
- Yeast cells with a disrupted *SLY2* gene are cold sensitive and show a defect in the glycosylation of secreted invertase at 15º C.

Work is in progress to localize the different *SLY* gene products within the cell and to isolate mutants of the essential *SLY1* and *SLY12* genes to unravel the function of these newly discovered genes.

H 334 CHARACTERIZATION OF SEC4P: A YEAST GTP BINDING PROTEIN REQUIRED FOR SECRETION, Alisa K. Kabcenell and Peter J. Novick, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510.

In Saccharomyces cerevisiae, the 24 kD GTP binding protein, Sec4p, is involved in the regulation of the polarized transport and fusion of post-Golgi secretory vesicles with the plasma membrane. Sec4p associated with the cytoplasmic aspect of vesicles can reach the plasma membrane through the exocytic pathway and recycle through a small soluble pool back to this compartment. Phenotypic analysis of mutations in SEC4 supports the hypothesis that superimposed on this cycle of protein localization is a cycle of guanine nucleotide binding and hydrolysis. The alteration in the conformation of vesicular Sec4p resulting from the binding of GTP could, for example, facilitate an interaction with a plasma membrane effector which would terminate upon hydrolysis of the bound nucleotide. In order to determine the mechanism of action of the protein, we have purified the soluble form of Sec4p from a yeast strain overproducing it. Sec4p binds GDP, GTP and triphosphate analogs in the presence of micromolar concentrations of free magnesium ions by exchange with prebound GDP. The enhanced stability of the GTP bound form of the protein is reflected in a 20-fold higher affinity for GTP over GDP, a decreased rate of nucleotide dissociation and an extremely low intrinsic GTPase activity. Based on the model of Sec4p function, it could be predicted that protein factors exist in vivo which could stabilize the inactive conformation of the protein, or stimulate nucleotide exchange or hydrolysis. In support of this, we have detected an activity in a yeast soluble fraction which will inhibit the binding of a radiolabeled triphosphate analog to Sec4p. Further characterization of this factor and its possible role in the Sec4p-mediated cycle of regulation of vesicular transport is currently underway.

H 335 A NOVEL GTP-BINDING PROTEIN, Sar1p, COOPERATES WITH Sec12p IN TRANSPORT FROM THE ENDOPLASMIC RETICULUM TO THE GOLGI APPARATUS, Akihiko Nakano and Shuh-ichi Nishikawa, Department of Biology, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

SAR1, a gene that has been isolated as a multicopy suppressor of a yeast ER-Golgi transport mutant *sec12*, encodes a novel GTP-binding protein. The predicted amino acid sequence (21 kD) contains complete consensus sequences of the GTP-binding domain of *ras* superfamily and a recombinant Sar1p produced in *E. coli* indeed binds GTP. Gene disruption experiments show that *SAR1* is essential for cell growth. To test its function further, a conditionally lethal, galactose-dependent mutant of *SAR1* has been constructed. This mutant accumulates ER-form precursors of α-factor and carboxypeptidase Y under the restrictive condition, indicating that *SAR1* is also required for ER-Golgi transport.

A polyclonal antibody against Sar1p has been raised using a lacZ-SAR1 fusion protein as an antigen. This antibody recognizes Sar1p as a 23-kD protein in yeast lysates. The protein sediments at a low speed centrifugation and is solubilized by detergent. Furthermore, subcellular fractionation experiments have shown that Sar1p and Sec12p cofractionate in both differential and sucrose density gradient centrifugation. We suggest that the two proteins cooperate in the early event of the ER-Golgi transport.

H 336 MOLECULAR ANALYSIS OF SEC12p, A MEMBRANE PROTEIN REQUIRED FOR PROTEIN TRANSPORT FROM THE ENDOPLASMIC RETICULUM TO THE GOLGI APPARATUS IN YEAST, Christophe d'Enfert and Randy Schekman, Department of Biochemistry, University of California, Berkeley, CA 94720

In order to better understand the mechanisms by which proteins are transported from the endoplasmic reticulum (ER) to the Golgi apparatus, we have focused our attention on Sec12p, a bitopic membrane glycoprotein that resides in the ER and Golgi membranes and whose function is necessary for the assembly of a vesicle intermediate in ER to Golgi transport. To study the topology of Sec12p, we have constructed a *SEC12-SUC2* gene fusion which encodes the N-terminal domain of Sec12p, its transmembrane domain and the *SUC2* encoded invertase. We have shown that the fusion protein bears the glycosylation characteristics of ER localized invertase. This suggests that the N-terminal domain of Sec12p is localized in the cytoplasm. Furthermore, we have shown that, when overproduced, this fusion protein as well as a truncated derivative of Sec12p, which lacks two thirds of the C-terminal domain, can replace the wild-type Sec12 protein. Therefore, the Sec12p C-terminal domain is dispensable for the transport function of Sec12p. This taken together with the fact that mutations which impair the Sec12p transport function affect the N-terminal cytoplasmic domain suggests that this domain is essential and could be the site of different protein interactions required for the formation of a vesicle intermediate. In this regard, we have shown that the overproduction in yeast of a soluble Sec12p cytoplasmic domain interferes with transport since it increases the defect of a *sec12* ts strain. We have also shown that this cytoplasmic domain can be produced in *Escherichia coli* in a soluble form and we are curently studying its potential use as an *in vitro* inhibitor of the transport process.

H 337 CELLULAR LOCALIZATION OF A FAMILY OF SMALL GTP-BINDING PROTEINS (THE RAB FAMILY), Bruno Goud[1], Ahmed Zaharoui[2], Nicolas Touchot[2] and Jaako Saraste[3], (1) Unite de Genetique Somatique, Institut Pasteur, Paris, France, (2) INSERM U 248, Faculte de Medecine Lariboisiere Saint-Louis, Paris, France, and (3) Ludwig Institute for Cancer Research, Stockholm, Sweden. Growing evidence suggests that small GTP-binding proteins (20-30Kd) may play a central role in the regulation of intracellular transport. In the yeast *Saccharomyces cerevisiae*, the genes YPT1 and SEC4 coding for GTP-binding proteins have been implicated in protein transport between the endoplasmic reticulum and the Golgi apparatus and between the Golgi apparatus and the plasma membrane respectively. In mammalian cells, a non-hydrolysable analog of GTP, GTPγS, blocks protein transport in various in vitro assays. Recently, several genes (the *rab* genes) coding for small GTP-binding proteins have been isolated from a human pheochromocytoma cDNA library. Rab1 p shows 75% amino acid identity with Ypt1 p and may be therefore considered as its human counterpart. The other proteins share about 40% homology with Ypt1 and Sec4 proteins (Zaharoui et al., 1989). We have produced these proteins in *Escherichia coli* and raised polyclonal antibodies against the purified proteins. The characterization of the antibodies against rab1,2,4,5 and 6 proteins is currently underway. By immunoblotting, these antibodies recognize a band at the predicted molecular weight of each GTP-binding protein. So far, the antibodies detect a band in all the lysates of mammalian cell lines we have screened, suggesting that the rab proteins are ubiquitous. At the steady state, the rab proteins are mostly membrane-bound but a small soluble pool exists for all of them. By immunofluorescence using affinity-purified antibodies, rab1,2,5, and 6 proteins are found to be located in the Golgi area of the cells. Anti-rab 4 antibodies stain filamenteous structures located around the nucleus. By electron microscopy, rab 6p is found to be associated with the cytoplasmic face of some Golgi cisternae. The localization of the members of the rab family indicate that they likely play a role in intracellular transport along the secretory pathway.

H 338 ADP-RIBOSYLATION FACTOR (ARF) IS A CONSTITUENT OF THE NON-CLATHRIN-COATED GOLGI TRANSPORT VESICLES; Tito Serafini, Michael Brunner, Richard A. Kahn*, and James E. Rothman; Dept. of Biology, Princeton University, Princeton, NJ 08544, and *Lab. of Biol. Chemistry, NCI, Bethesda, MD 20892. GTP-blotting experiments have revealed that several cytosolic low molecular weight GTP-binding proteins (LMW GTPBP's) accumulate on Golgi membranes during our *in vitro* intra-Golgi transport reaction. These proteins are not appreciably present on the membranes prior to their inclusion in the reaction, and significant binding occurs only in the presence of the non-hydrolyzable GTP analogue GTPγS during incubation at 37 °C, conditions which also cause an accumulation of non-clathrin-coated transport vesicles. An improved vesicle purification allows the assignment of at least four LMW GTPBP's as vesicle constituents; using immunoblotting, one vesicle LMW GTPBP has been shown to be ADP-ribosylation factor (ARF). ARF, an abundant protein present in a wide variety of tissues, was originally identified as a cofactor required in the cholera-toxin-catalyzed ADP-ribosylation of the alpha subunit of G_S. Preliminary studies indicate that ARF is enriched in the vesicles relative to incubated Golgi membranes and is present at many molecules per vesicle. Thus ARF is the first identified mammalian LMW GTPBP found to be a transport vesicle component. Together with other data indicating a role for ARF in protein secretion in *S. cerevisiae* (Tim Stearns, Mark C. Willingham, David Botstein, and R.A.K., 1989 CSH Yeast Cell Biology Meeting abstract), our findings indicate a probable role for ARF in intracellular protein transport.

H 339 THE MECHANISM OF GLYCOLIPID TRANSPORT THROUGH THE GOLGI IS BIOCHEMICALLY AND KINETICALLY INDISTINGUISHABLE FROM THAT OF GLYCOPROTEIN TRANSPORT. A CELL FREE SYSTEM FOR MEASURING GLYCOLIPID TRANSPORT TO THE TRANS GOLGI. Binks W. Wattenberg, Cell Biology Unit, The Upjohn Company, Kalamazoo, MI 49001. While the mechanisms of protein transport to the cell surface are being extensively studied, the tools for study of lipid transport have not been as well developed. Here I describe the formulation of a cell free assay which measures the intercompartmental transport of a glycolipid to the trans Golgi. "Donor" Golgi membranes are derived from the glycosylation mutant Lec2, deficient in the Golgi CMP-sialic acid transporter. In these cells the major glycolipid is lactosyl-ceremide. The "acceptor" Golgi are derived from Lec8 cells, deficient in the UDP-Gal transporter, where the major glycolipid is glucosyl-ceremide. The transport of lactosyl-ceremide from donor to acceptor results in the formation of the ganglioside GM_3 (sialic acid-lactosyl-ceremide). The transport reaction is dependent on ATP, elevated temperature, intact Golgi membranes, cytosolic proteins, and is inhibited by GTPγS. Furthermore the reaction depends on the N-ethylmaleimide sensitive factor previously shown to participate in protein transport from the endoplasmic reticulum to the Golgi, through the Golgi, and in endocytosis. The transport of glycolipid and protein was measured in the same set of membranes, allowing precise comparison of the kinetics of transport to the trans Golgi. The kinetics of transport were identical for protein and lipid transport. These data indicate that glycoproteins and glycolipids are transported through the Golgi by similar or identical mechanisms.

H 340 ISOLATION AND CHARACTERIZATION OF SUPPRESSORS OF THE SECRETION MUTATION sec14-1^{ts}, Kathleen M. Champion, Ann E. Cleves, and Vytas A. Bankaitis, Department of Microbiology, University of Illinois, Urbana, IL 61801. Yeast bearing the sec14-1^{ts} mutation suffer Golgi-associated secretory defects as a result of dysfunction of the SEC14p, an essential cytosolic factor that somehow promotes Golgi secretory function. In order to identify both targets and antagonists of SEC14p function, we independently isolated 107 spontaneously arising revertants of sec14-1^{ts} haploid strains. Genetic analyses demonstrated that all of these suppressor mutations were extragenic in nature and that 26 of these identified recessive suppressor mutations. Complementation analysis has revealed that the recessive suppressors define four genes: sac1, rsd2, rsd3, and rsd4. These genes identify potential antagonists of SEC14p function. Linkage analysis of the 81 dominant suppressor mutations has demonstrated the existence of two genes: dsd1, represented by 80 mutants, and dsd2, represented by one mutant. The dominant suppressors represent potential targets of SEC14p function.

Although the sec14-1^{ts} defect can be dosage compensated, the mechanism of suppression is not by up-regulation of sec14-1^{ts}. In fact, all of the pseudorevertants are capable of suppressing disruptions of the sec14 gene. The suppressors fully compensate for both the temperature-sensitive and disrupted alleles of sec14, allowing complete restoration of growth and secretion under otherwise nonpermissive conditions. Although the suppressors bypass sec14, they appear to do so through the normal pathway of secretion.

H 341 THE RSD4 GENE PRODUCT IS AN ANTAGONIST OF YEAST GOLGI SECRETORY FUNCTION, Vytas A. Bankaitis and Todd McGee, Department of Microbiology, University of Illinois, Urbana, IL 61801. The yeast RSD4 gene was originally identified by two non-complementing mutations that exhibited a recessive suppression of yeast sec14-1^{ts} Golgi secretory defects. We have employed the phenotypic properties of rsd4, sec14-1^{ts} mutants to recover a genomic clone of the RSD4 gene. Gene disruption experiments indicated that RSD4 was not essential for yeast vegetative growth. Moreover, the putative rsd4° alleles were efficient suppressors of sec14-1^{ts} and recessive-lethal sec14-129::H1S3 mutations. These suppressor strains exhibited essentially wild-type secretory capabilities, and secretion appeared to occur via the normal route. The epistatic relationship of rsd4° mutations to sec14 mutations is consistent with a general model where the RSD4 gene product is a negative regulator of Golgi secretory function. We propose that the SEC14p is a stimulatory factor that participates in a regulatory pathway which controls the movement of secretory proteins from the yeast Golgi. We envision the RSD4 gene product to exhibit an execution point in such a pathway that lies downstream of the SEC14p execution point.

H 342 ENZYMOLOGY OF PROTEIN TRANSPORT BETWEEN THE GOLGI CISTERNAE. Vivek Malhotra and James E Rothman. Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544. Transport of Proteins between the Golgi cisternae is mediated by non-clathrin coated vesicles. These vesicles have to be uncoated before the fusion with Golgi cisterna can take place (1,2,). We have developed a scheme for the purification of these Golgi-derived coated vesicles (3). Electron microscopy has revealed that incubation of coated vesicles with 1M KCl results in the uncoating of vesicles. The major polypeptides released from the vesicles with 1M KCl consists of a polypeptide of 160 kD and a series of proteins in the range of 100 kD. The release of these polypeptides from coated vesicles is currently being used as an assay to identify the physiological activity responsible for the uncoating of Golgi-derived coated vesicles.

1. Malhotra, V., Orci, L., Glick, B., Block, M and Rothman, J.E. (1988). Cell 54,221-227
2. Orci, L., Malhotra, V., Amherdt, M., Serafini, T and Rothman, J.E. (1989). Cell, 56,357-368.
3. Malhotra, V., Serafini, T., Orci, L., Shepherd, J. and Rothman, J.E. (1989). Cell, 58,329-336

H 343 POSSIBLE INVOLVEMENT OF A CALMODULIN-LIKE PROTEIN IN REGULATING TRANSPORT THROUGH THE GOLGI STACK, Mitsuo Tagaya and James E. Rothman, Department of Biology, Princeton University Princeton, NJ 08544. Addition of 30 μM calmodulin (CaM) to the cell-free Golgi transport system in the presence of Ca^{2+} almost completely inhibited transport. Inhibition was reversed by EGTA. This raises the possibility that CaM may regulate protein transport through the Golgi stack by shutting down constitutive secretion during Ca^{2+}-induced, regulated secretion. Kinetic studies suggest that Ca^{2+}-CaM inhibits at a stage after vesicles are attached to the acceptor membane, but before vesicle-acceptor membrane fusion . CaM antagonists also inhibited transport in a similar manner to CaM. Inhibition by the antagonists is independent of calcium ion. A working hypothesis is that a calmodulin-like protein is needed for constitutive transport. This protein would be inhibited by CaM antagonists, and would bind to its target independent of calcium. Ca^{2+}-CaM may act as a competitive inhibitor of transport by binding to the target site in an ineffective manner.

H 344 USE OF p58 AS A MARKER TO IDENTIFY VESICULAR COMPONENTS OPERATING IN MEMBRANE TRAFFIC BETWEEN THE ER AND GOLGI, Jaakko Saraste, Ulla Lahtinen, and Kerstin Svensson, Ludwig Institute for Cancer Research, Box 60202, S-10401 Stockholm, Sweden. Immunolocalization in light and electron microscopy with antibodies to a 58 kD protein (p58) was used to obtain information of the elements which function in protein transport between the ER and Golgi. p58 is an integral membrane protein and appears to be a major component of the pre-Golgi membranes. It is present in cells in dimeric and larger (probably hexameric) form. In NRK cells grown at 37°C p58 was found in tubulovesicular elements seen in the Golgi region and in cis Golgi and occasionally also in the cell periphery. Some p58 was also detectable in the ER. In cells shifted to low temperature (16°C) the accumulation of a large number of these p58-containing peripheral elements was observed. They were shown to be intermediates in biosynthetic protein transport by colocalizing p58 and a viral membrane protein, arrested between the ER and the Golgi complex at 15°C, in BHK-21 cells. Low temperature apparently causes the accumulation of pre-Golgi elements by slowing down their movement since if the cells were returned to 37°C a rapid translocation of these elements to the Golgi region was observed. Nocodazole inhibited this translocation process suggesting that the movement of pre-Golgi elements requires the integrity of microtubules. Brefeldin A (BFA), a fungal antibiotic which blocks protein transport between the ER and Golgi complex, induced the redistribution of p58 to reticular ER elements (including the nuclear membrane). In the presence of BFA p58 also accumulated in tubulovesicular structures throughout the perinuclear ER region which apparently represent the sites where BFA affects protein transport. These results indicate that the transport of proteins from ER to Golgi is not restricted to a region adjacent to the Golgi complex but occurs from multiple transitional sites within the perinuclear ER network. As our data suggests that p58 may continuously cycle between the ER and Golgi, we are currently using immunoelectron microscopy to possibly identify the elements which function in the recycling process.

H 345 ER TO GOLGI TRANSPORT IN *sec13* AND *sec19* IS TEMPERATURE-SENSITIVE IN VITRO AND CAN BE RESCUED BY THE ADDITION OF WILD TYPE CYTOSOL. Nancy K. Pryer, Chris A. Kaiser, and Randy W. Schekman, Dept. of Molecular and Cell Biology, Univ. of California, Berkeley, CA 94704.

We are studying genes involved in two different steps of transport between the endoplasmic reticulum and the Golgi complex. The temperature-sensitive yeast mutants *sec19-1*, *sec13-1* and *sec13-4* are defective in ER to Golgi transport at the non-permissive temperature. *sec13* cells accumulate exaggerated ER structures, suggesting that the *SEC13* gene product is required for the formation of a vesicular transport intermediate. In contrast, *sec19* cells accumulate a variety of membranous structures including ER and Golgi membranes and secretory vesicles. We have reconstituted the *sec13* and *sec19* defects in vitro using an ER to Golgi transport assay consisting of membrane and cytosolic fractions prepared from mutant cells. The efficiency of ER to Golgi transport at the permissive temperature in this assay is reduced four to five-fold by preincubation of *sec19* cytosol at 30°C or *sec13* cytosol at 36°C. Preincubation of membrane components has little effect on transport, suggesting that the *sec19* and *sec13* defects are primarily cytosolic. In addition, both the *sec19* and *sec13* defects can be rescued completely by the addition of wild type cytosol to mutant membranes. Complementation of the ER to Golgi transport defect in vitro will be used as an activity assay in the purification of the Sec13 and Sec19 proteins. In order to better characterize the mutations, we have also cloned the *SEC13* and *SEC19* genes. The *SEC13* gene is 1.3 kB in length. Antibodies raised against a a Sec13 fusion protein recognize a 34kD protein that is overproduced in wild type stains carrying a multicopy plasmid containing the *SEC13* gene. We have recently isolated a 5 kB DNA fragment that rescues both temperature-sensitive growth and ER to Golgi transport in *sec 19* when present on a single copy plasmid. We are currently characterizing the putative *SEC19* gene using molecular genetic techniques.

H 346 REVERSIBLE DISORGANIZATION OF THE GOLGI COMPLEX MODULATED BY BREFELDIN A. Yukio Ikehara, Toshiyuki Fujiwara and Kimimitsu Oda, Department of Biochemistry, Fukuoka University School of Medicine, Fukuoka 814-01, Japan.

We reported that brefeldin A(BFA) blocks the protein transport from the endoplasmic reticulum(ER) to the Golgi complex [J. Biol. Chem. 261, 11398(1986)] and causes rapid disassembly of the Golgi complex and accumulation of secretory proteins in the ER [J. Biol. Chem. 263, 18545(1988)]. In this study we further examined the effect of BFA on the distribution of the Golgi marker thiamine pyrophosphatase(TPPase) in rat hepatoma cells. Cytochemical electron microscopy of the cultured cells demonstrated that TPPase activity was localized in the Golgi complex. When the cells were treated with BFA (2.5 μg/ml) for 10 min, the characteristic Golgi stack was no longer detectable, and TPPase was cytochemically stained in vesicular and tubular structures scattered in the cytoplasm. Longer incubations with BFA (20 min to 1 h) resulted in distribution of the enzyme activity in the ER and nuclear envelope. Such unusual distibution of the enzyme activity, however, was reversible even in the presence of BFA. At 2 h after the treatment, the TPPase activity disappeared from the ER and was concentrated again in the vesicular/tubular structures. It was finally localized in the Golgi complex reassembled by 4 h after the exposure. The reversible effect of BFA may be due to a possible metabolism of the drug into an inert form during the incubation. Taken together, these results indicate that BFA causes rapid disassembly of the Golgi complex and redistribution of the marker enzyme TPPase into the ER. The spontaneous reversibility of the drug effect favors a dynamic recycling of the Golgi marker between the ER and the Golgi complex under the conditions tested here.

H 347 **COLLIGIN/HSP47 IS DOWN REGULATED BY BREFELDIN A IN LIGAMENT AND BONE CELLS.** John Sauk, Kathy Norris, Ruth Foster and Martha Somerman, Departments of Pathology and Pharmacology, University of Maryland Dental School, Baltimore, MD 21201.
Brefeldin A is an antiviral drug capable of rapidly and reversibly blocking the transport of newly synthesized proteins out of the ER. This change is accompanied by a redistribution of cis/medial Golgi components to the ER. Proteins retained in the ER undergo processing by these recycled cis/medial Golgi enzymes, but do not acquire sialic acid, a modification mapped to the trans Golgi system (Lippincott-Schwartz et al. *Cell* 56: 801, 1989). The characteristics of this drug provide an opportunity to inhibit internal membrane transport to examine the expression of resident ER proteins. Colligin/Hsp47, a collagen binding membrane glycoprotein believed to function in protein sorting (Nagata et al., 1988), was studied. Osteoligament cells and *ROS 17/2.8* cells were grown in culture and treated with Brefeldin A (Sandoz, Switzerland). Total cellular and secreted protein and collagen were determined after labeling with ^{3}H-Proline (Peterkofsky, 1971). Levels of colligin were ascertained by immunoprecipitation utilizing anti-colligin antibodies (*courtesy, K. Yamada, NIH, NCI*). These results show that total protein and collagen secretion is inhibited with intracellular accumulation during Brefeldin A treatment. Accumulation of intracellular proteins was associated with down regulation of the levels of Colligin/Hsp47. These data support the role of colligin/Hsp47 in protein sorting. Supported by: **DE-08648, DE-07512, NIH, NIDR.**

H 348 PROTEIN EGRESS FROM THE ENDOPLASMIC RETICULUM IS NECESSARY FOR ENDOGENOUS ANTIGEN PRESENTATION, Jed G. Nuchtern*, William E. Biddison+, and Richard D. Klausner*, *Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development and +Neuroimmunology Branch, National Institute of Neurologic Disorders and Stroke, NIH, Bethesda, MD 20892. Cytosolic antigens must be partially degraded, transported into the lumen of the vacuolar system and form complexes with MHC molecules before they can be presented to the antigen receptor on cytolytic T lymphocytes (CTL). For class I restricted antigens this process is insensitive to inhibitors of lysosomal degradation, such as chloroquine, and blocked by inhibitors of protein synthesis, thus it has been called endogenous to distinguish it from the exogenous pathway in which soluble protein antigens are taken up into endosomes where they are partially degraded and form complexes with MHC molecules. Using the drug Brefeldin A we have previously shown that the endogenous pathway of class I restricted presentation is dependent on protein transport out of the endoplasmic reticulum (ER). These results imply that the ER may be the site where class I MHC and processed antigen first combine. Previous attempts to define an endogenous pathway for a class II restricted cytoplasmic antigen, such as Influenza A matrix protein, have been hindered by an inability to establish an infection in the absense of endocytosis and endosomal acidification. When normal endocytosis is allowed to take place, exogenous antigen processing also occurs. In order to delineate a non-endosomal, endogenous pathway for presenting this cytosolic antigen, we used a technique which allows direct fusion of virus particles with the presenting cell's plasma membrane. Under these conditions matrix protein is presented to class II restricted CTL in a chloroquine-insensitive manner. This process is blocked by Brefeldin A, implying a strict requirement for protein transport from the ER in this process and suggesting that the ER may in fact be the site of antigen-MHC assembly in class II restricted endogenous antigen presentation as well.

H 349 MICROTUBULE-DEPENDENT RETROGRADE TRANSPORT OF PROTEINS INTO THE ER IN THE PRESENCE OF BREFELDIN A REVEALS AN ER RECYCLING PATHWAY, Jennifer Lippincott-Schwartz, Julie G. Donaldson, Hans-Peter Hauri*, Lydia Yuan and Richard D. Klausner, Cell Biology and Metabolism Branch, National Institutes of Health, Bethesda, MD and *Biozentrum, Basel, Switzerland.
The characteristics of Brefeldin A (BFA)-induced retrograde movement of Golgi proteins into the ER and its relationship to an ER retrieval pathway were investigated. Retrograde movement of Golgi into the ER occurred by a mechanism involving the extension of numerous, long, tubulo-vesicular processes out of the Golgi along microtubules which then fused with the ER. Microtubule disrupting agents (i.e. nocodazole), energy poisons and reduced temperatures (<16^{0}C) inhibited this pathway. Although pretreatment of cells with nocodazole blocked retrograde movement of Golgi proteins, if these proteins were first transported to the ER by BFA, subsequent addition of nocodazole had a surprising effect: the ER was cleared of the Golgi markers which accumulated in structures which co-localized with a 53 kD protein normally associated with intermediate transport vesicles between the ER and Golgi. These results were consistent with the movement of Golgi proteins in the presence of BFA through an ER cycling pathway whose retrograde arm is microtubule-dependent. To determine whether such an ER cycling pathway is followed by any proteins in the absence of BFA, we studied the dynamics of the 53 kD marker. At 16^{0}C, the 53 kD protein redistributed into a central structure closely associated with the Golgi and into long, tubulo-vesicular processes extending out of it. Upon addition of BFA at 16^{0}C Golgi proteins entered the tubular processes marked by the 53 kD protein. These results suggested that BFA induces the interaction of the Golgi with a nearby intermediate, recycling compartment enabling the Golgi to enter retrograde tubulo-vesicular processes that leads to fusion with the ER.

Genetic and in Vitro Analysis of Cell Compartmentalization

Membrane Proteins, Lipids and Topogenesis; Yeast Secretion and Clathrin; Nuclear Assembly; Cytoskeleton

H 400 LIPOPROTEIN EXPRESSION IN *SACCHAROMYCES CEREVISIAE*, Stephen L. Sturley and Alan D. Attie, Dept. of Biochemistry, University of Wisconsin, Madison, WI 53706. Apolipoprotein E (apo-E) and B (apo-B) are the major protein components of mammalian very low density (VLDL) and low density (LDL) lipoproteins. They maintain the structural integrity of these particles and mediate their clearance from the vascular system by receptor mediated endocytosis. The mechanisms that lead to the formation of these lipid rich particles are of relevance to the causes of hypercholesterolemia as well as being of intrinsic interest. We have expressed the apo-E gene and domains of the apo-B gene in the simple eukaryote *S. cerevisiae*, and are developing a novel system for the analysis of lipoprotein particle assembly. We have found that secretion of apo-E by yeast requires a host mutation conferring sterol uptake (*upc2*, uptake of cholesterol). Contrary to our expectations, secretion occurs only when the mutant cells are grown in the absence of sterol. Wild-type or *upc2* strains repleted of sterol do not secrete apo-E. We propose that the *upc2* defect relieves a blockage to lipoprotein secretion and that this deficiency is overcome by the provision of sterol. Similarly, the putative receptor binding domains of apo-B are secreted at higher levels in the *upc2* host deprived of sterol than in wild-type strains. The secreted polypeptides bind to the LDL receptor and therefore are presumed to be in an active conformation. Additionally, we have expressed N-terminal regions of apo-B that have a strong affinity for lipid and are currently analysing particle formation by these polypeptides. This combination of classical and molecular yeast genetics is providing a unique opportunity to analyse lipoprotein biogenesis.

H 401 SACCHAROMYCES CEREVISIAE STE6 GENE PRODUCT: EVIDENCE FOR A NOVEL PATHWAY OF PROTEIN EXPORT IN EUKARYOTIC CELLS, Karl Kuchler, Rachel E. Sterne and Jeremy Thorner, Division of Biochemistry and Molecular Biology, University of California, Berkeley, CA-94720.

MATa haploids of the yeast *S. cerevisae* release a lipopeptide mating pheromone, called a-factor. The precursors of a-factor (MFa1 and MFa2 gene products) are very short, lack a cleavable hydrophobic signal sequence and do not possess consensus sites for Asn-linked oligosaccharides. Secretion of mature a-factor can occur at non-permissive temperature in MATa cells that carry temperature-sensitive secretion-defective mutations (sec). Using anti-a-factor antibodies and subcellular fractionation experiments, we could show that newly synthesized a-factor resides in the cytoplasm, suggesting that a-factor is processed and released from MATa cells by a route that is quite distinct from the classical secretory pathway.
Mutations have been isolated that prevent a-factor secretion. MATa ste6 haploids fail to release mature a-factor, but accumulate a-factor related peptides intracellularly. Gene dosage experiments using multi-copy plasmids carrying the STE6 gene and the MFa1 gene, indicated that STE6 gene product is rate-limiting for a-factor production. DNA sequence analysis revealed that STE6 encodes a large transmembrane protein which bears extensive homology to a superfamily of prokaryotic transport proteins (including hlyB, hisP, malK, oppD, oppF and pstB). More strikingly, STE6 protein is most similiar, both in amino acid sequence, length and predicted transmembrane topology, to a known mammalian plasma membrane-bound transporter, the mdr (multiple-drug-resistance) protein. The data suggest that STE6 protein in yeast, and possibly mdr-like proteins in animal cells, are part of a novel pathway for the transport of peptides or proteins in eukaryotic cells.

H 402 pKD1-BASED SECRETION VECTORS FOR THE PRODUCTION OF HUMAN RECOMBINANT PROTEINS IN KLUYVEROMYCES YEASTS, Patrice Yeh, Reinhard Fleer, Xin J. Chen*, Isabelle Maury, Nacima Faulconnier, Jean D. Guitton, Hiroshi Fukuhara*, and Jean-François Mayaux, Biotechnology Department, Rhône Poulenc Santé, BP 14, 94403 Vitry Cedex and *Institut Curie, Centre Universitaire, 91405 Orsay, France.

Using multi-copy secretion vectors based on a cryptic plasmid (pKD1) initially isolated from K. drosophilarum (Chen X.J. et al., Nucl. Acids Res. 14 (1986) 4471-4481) we expressed a variety of human genes under control of natural or hybrid yeast promoters in yeasts of the genus Kluyveromyces. Secretion of immunologically reactive material and/or enzymatically active proteins was achieved with human serum albumin (HSA), interleukin-1ß (IL-1ß), 1^{rst} and 2nd generation tissue plasminogen activators (tPA), and tissue specific inhibitor of metalloproteinases (TIMP). Except for IL-1ß whose secretion was directed by the signal peptide of the K. lactis killer toxin α subunit, it is demonstrated that the natural human exportation signals from these proteins are functional in Kluyveromyces. In addition, analysis of the N-terminal sequence of secreted HSA and IL-1ß revealed equally efficient removal of the signal sequence, regardless whether a human or a yeast signal was used. Whereas expression of Met-tPA and Met-IL-1ß yielded unglycosylated proteins accumulating in the cytoplasm, heavily glycosylated material was obtained when expressing prepro-tPA, pre-TIMP and pre-IL-1ß. Glycosylation of recombinant IL-1ß (which does not occur in mammalian cells) causes the loss of 90 % of its biological activity as monitored in a lymphocyte activation assay and this activity can be fully restored by treatment with endoglycosidase H. The fact that recombinant HSA which is devoid of carbohydrate residues is secreted at levels well above those observed with IL-1ß, TIMP or tPA raises the possibility that hyperglycosylation might interfere with efficient protein secretion in Kluyveromyces yeasts.

H 403 POSTTRANSLATIONAL PROCESSING AND INTRACELLULAR LOCALIZATION OF THE YEAST KEX2 PROTEASE, AN ENZYME THAT CLEAVES PRECURSORS AT PAIRED BASIC RESIDUES, Robert S. Fuller, Celeste Wilcox and Kevin Redding, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305. Analogous to cleavage of prohormones and neuropeptide precursors such as pro-insulin and proopiomelanocortin, the yeast (*Saccharomyces cerevisiae*) Kex2 protease cleaves pro-α-factor and pro-killer toxin, during their transit of the secretory pathway, at the carboxyl side of Lys-Arg and Arg-Arg dipeptides. In order to identify the intracellular site of processing in yeast, we have characterized posttranslational modifications of the protease and examined its intracellular distribution using indirect immunofluorescence. Pulse-chase/immuno-precipitation studies, combined with analysis of carbohydrate and of secretory (*sec*) mutants, demonstrate that Kex2 protease undergoes rapid (≤ 1 min) Asn- (N-) and Ser/Thr- (O-) glycosylation and signal peptide cleavage upon translocation into the ER. A second proteolytic processing step occurs within 1-2 min, requires prior signal peptidase action and corresponds to removal of an amino-terminal pro-peptide of about 9-10 kDa. Subsequent transport to the Golgi occurs within 4 min and is marked by extension of O-linked oligosaccharide. This is followed by a slow increase in M_r due to addition of α-1,3-linked mannose residues to O-linked chains. The latter result shows that for the duration of the lifetime of the enzyme ($t_{1/2}$~ 90 min), mature Kex2 protein is accessible to the α-1,3-linked mannosyl transferase which is thought to reside in a late compartment of the Golgi. Indirect immunofluorescence shows that, consistent with previous results [R.S. Fuller, A. Brake and J. Thorner (1989) Science, in press], Kex2 protein is an intracellular molecule. The protein is localized in a punctate fashion to multiple bodies (3-10/cell) that are distributed throughout the cytoplasm of the yeast cell, without association with the nucleus or other identifiable organelle. Thus yeast Golgi, unlike that of higher eukaryotes, appears to comprise a dispersed set of compartments rather than a single perinuclear structure. Supported by a Lucille P. Markey Scholar award and NIH grant GM39697 to R.S.F., an NIH predoctoral fellowship to C.W. and an NSF predoctoral fellowship to K.R.

H 404 GENETICS OF YEAST STRAINS IN WHICH CLATHRIN-DEFICIENCY IS LETHAL. Alan L. Munn, Marc Elgort, and Gregory S. Payne, Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024 and Linda Silveira, Department of Biochemistry, University of California at Berkeley, Berkeley, CA 94720 In the majority of *Saccharomyces cerevisiae* strains which have been examined, an extensive deletion of the gene encoding clathrin heavy chain (*CHC1*) is not lethal to the cell, but causes slow growth on rich media. One laboratory strain of *Saccharomyces cerevisiae* has been found, however, in which deletion of the *CHC1* gene (*Δchc1*) causes lethality. The allele responsible for lethality of *Δchc1* in this strain, which is unlinked to *CHC1*, has been called *SCD1* (*suppressor of clathrin deficiency*)[1]. Recently, a second strain (W303) has been found in which the *Δchc1* mutation is lethal and we are currently investigating whether this lethality is due to a single allele. Studies in this lab have been undertaken using a construct in which *CHC1* expression is controlled by the repressible *GAL1* promoter and these studies have shown that the *GAL1-CHC1* construct spares *Δchc1* strains from the lethality associated with *SCD1* and the allele(s) in W303, even under conditions where the *GAL1* promoter is repressed. The growth of the *SCD1* and W303 strains carrying *Δchc1* and *GAL1-CHC1* is, however, extremely poor under repressive conditions. The extremely poor growth phenotype associated with both *SCD1* and the W303 allele(s) in *Δchc1GAL1-CHC1* backgrounds is recessive to the slow growth phenotype of alleles present in strains which survive *CHC1* deletion, and preliminary complementation and segregation data suggest that *SCD1* and the allele(s) in W303 may affect distinct genes.

1. Lemmon, S.K., and Jones, E.W. (1987) *Science* 238: 504-509.

H 405 ISOLATION AND CHARACTERIZATION OF YEAST MUTANTS WITH TEMPERATURE-SENSITIVE CLATHRIN HEAVY CHAINS, Judith A. Finlay, Babak Pishvaee and Gregory S. Payne, Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024

Mutant yeast cells with a deletion of the clathrin heavy chain (*CHC*) gene, among other defects, grow more slowly than wild type cells, secrete a high molecular-weight form of unprocessed α-factor, and mislocalize Kex2 endoprotease to the cell surface. To distinguish between primary defects caused by the clathrin deficiency and secondary defects that might arise after deletion of the gene, a temperature-sensitive (ts) mutation in the *CHC1* gene was generated by *in vitro* mutagenesis with hydroxylamine. The mutation responsible for temperature-sensitivity has been mapped to the 3' end of the gene. This region of the gene is now being sequenced. The ts mutation was introduced into the chromosomal *CHC1* gene and the resulting strain was characterized. At 23°C, both stains had a doubling time of 2.4 h. When cells were shifted to 37°C, five hours elapsed before a growth defect became apparent in the mutant cells. At this time the mutant cells had a doubling time of 2.1 h compared to 1.5 h for wild type cells. Maturation of α-factor was assessed after mutant cells were subjected to a 30 min preincubation and then a 30 min metabolic labeling with [^{35}S] amino acids at 37°C. Immuneprecipitation of secreted α-factor revealed that the ts strain secreted only unprocessed α-factor and no mature α-factor. Since the defect in α-factor processing occurred long before a defect in growth was detected, these data imply that mislocalization of Kex2 endoprotease and the resulting secretion of high molecular weight, unprocessed α-factor by clathrin-deficient strains occurs rapidly after loss of clathrin function.

H 406 CONSERVATION AND DIVERSITY IN COATED VESICLE ADAPTINS, Sreenivasan Ponnambalam[1], Margaret S. Robinson[2], Antony P. Jackson[1], Laurence Peiperl[1] and Peter Parham[1]. [1]Department of Cell Biology, Stanford University School of Medicine, Stanford, CA 94305 and [2]Cell Biology Division, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.
Coated pits and vesicles are dynamic membrane organelles responsible for receptor-mediated pathways of intracellular transport. Clustering of receptor-ligand complexes at the plasma membrane is associated with the co-polymerization of two classes of proteins on the cytoplasmic face of these organelles. The outer layer is comprised of triskelions containing clathrin heavy and light chains and the inner layer is a complex of assembly polypeptides which are thought to bind to receptors and facilitate the assembly of clathrin. Prominent among these assembly polypeptides are a heterogenous group of 100kd polypeptides, called adaptins, that are postulated to be adaptor molecules which conjoin receptors to clathrin. Three distinct adaptin families (α, ß, γ) have been characterized. They form two adaptor complexes that localize to distinct regions of the cell: one to the Golgi complex, the other to the plasma membrane. We have cloned the cDNA encoding the ß adaptin of the plasma membrane adaptor. The predicted structure of the 937 amino acid protein is identical between rat and human. This remarkable conservation contrasts with the absence of significant sequence similarity between the α and ß families of adaptins. Diversity within each adaptin family is created by the expression of different genes and by tissue-specific differential splicing. The structure of ß and α adaptins can be divided into two globular domains connected by a variable and potentially flexible stalk domain.

H 407 CLATHRIN LIGHT CHAIN TURNOVER AND DISTRIBUTION, Susan L. Acton and Frances M. Brodsky, Department of Pharmaceutical Chemistry, University of California, San Francisco, 94143. The clathrin light chains, LCa and LCb, have properties which suggest that they may be regulatory elements of clathrin assembly and/or disassembly, however their exact functions are unknown. Studies in a human B lymphocyte cell line have revealed that much more LCa than LCb is synthesized in a four hour period (Brodsky, JCB 101:2055 (1985)). To determine if LCa has a more rapid rate of turnover than LCb, the half-lives of the clathrin subunits were determined by pulse-chase studies. Half-lives of 24 hours, 45 hours, and 49 hours were determined for LCa, LCb, and the heavy chain, respectively. These results indicate that all clathrin subunits are sufficiently long-lived to undergo numerous rounds of endocytosis. Additionally, since the half-life of LCa is about half that of both LCb and the heavy chain, it is unlikely that the clathrin triskelion turns over strictly as a unit. Although LCa has a considerably shorter half-life than LCb, the difference in their half-lives is insufficient to explain the very large difference in their rates of synthesis. Quantitative studies of the light chains confirmed that this B lymphoctye cell line expresses much more LCa than LCb (approx. a 6:1 ratio). This finding prompted further investigation of the ratios of light chains in different tissues and cell lines. Of the tissues tested, most revealed a 1:1 LCa to LCb ratio. Brain tissue was the major exception, having at least twice as much LCb as LCa. In contrast to the tissues, the two cell lines tested showed a very large LCa to LCb ratio (approx. 6:1). Preliminary results indicate that the light chain ratios of assembled clathrin are the same as that of total cellular clathrin. (Supported by NIH grant GM30893 and NSF grant DCB8711317).

H 408 LOCALIZATION OF THE MEMBRANE-ASSOCIATED REGION OF VESICULAR STOMATITIS VIRUS M PROTEIN AT THE N-TERMINUS BY ^{125}I-TID LABELING, John Lenard and Roger Vanderoef, Department of Physiology and Biophysics, UMDNJ-Robert Wood Johnson Medical School (at Rutgers), Piscataway, N.J. 08854-5635.
^{125}I-TID has been successfully used to label amino acid residues that penetrate biological membranes. To identify the membrane-penetrating region of vesicular stomatitis (VSV) M protein, ^{125}I-TID was added to purified virions, and the mixture illuminated to activate covalent bond formation. Under these conditions, lipid, G and M proteins were significantly labeled, in a ratio of 16:1:0.4. Most of the lipid and G protein were extracted with Triton-X-100 at low ionic strength. The M protein, in association with the nucleocapsid, was subjected to trypsin digestion, which cleaves M protein in several steps from the N-terminus to residue 42. The M protein fragments produced by brief incubation, which were missing only 4-9 residues from the N-terminus, were essentially devoid of label, indicating that this N-terminal peptide consititutes the membrane penetrating region of M protein. This localization was confirmed by cleavage of purified M protein at asp-pro linkages (residues 54-55) with formic acid, and by cleavage at tryptophan residues with N-chlorosuccinimide. An amphipathic helix of 2-3 turns is proposed as the membrane-penetrating structure.

H 409 ROLE OF BETA-2 MICROGLOBULIN (β_2m) AND PEPTIDE LIGAND IN THE EXPRESSION OF CLASS I MAJOR HISTOCOMPATIBILITY MOLECULES.
Nancy Myers, Ted Hansen, Nancy Johnson and Wen-Rong Lie, Department of Genetics, Washington University School of Medicine, St. Louis, MO. It has recently been shown that class I molecules function as receptors that bind peptide ligands, thus potentiating the immune recognition of virus-infected or malignant cells by cytotoxic T cells. Considerable evidence supports the role of β_2m in the conformation, intracellular transport or surface expression of class I molecules. However, the precise nature and critical time that β_2m must interact with class I has not been defined. Futhermore, Townsend et al. recently reported evidence suggesting peptide ligand facilitates the assembly of class I with β_2m. To dissect the various factors influencing class I synthesis, we are studying the L^d class I molecule of the mouse. Our findings indicate that increased production of β_2m does not significantly alter L^d conformation or surface expression. However, culturing cells in the presence of an L^d-specific peptide led to a substantial increase in surface expression of L^d molecules. These findings underscore the importance of the peptide ligand in the de novo synthesis, transport and surface expression of class I molecules.

H 410 DETERMINANTS OF MEMBRANE PROTEIN TOPOLOGY. Dana Boyd[1,2] and Jon Beckwith[1]. [1]Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston MA 02115. [2]Department of Molecular Biology, University of California, Berkeley CA 94720.
We have used the alkaline phosphatase fusion technique to characterize determinants of membrane protein topological structure. When alkaline phosphatase is fused to the periplasmic domain of a cytoplasmic membrane protein, it is effectively exported to the periplasm. Such a hybrid protein exhibits high alkaline phosphatase activity. When alkaline phosphatase is fused to the cytoplasmic domain of a membrane protein, it remains, for the most part, in the cytoplasm. Such fusions exhibit low enzymatic activity. However, stable retention of alkaline phosphatase in the cytoplasm requires the presence in the fusion protein of the cytoplasmic loop ordinarily present in that position in the native, unfused protein. We have used oligonucleotide-directed mutagenesis to show that that positively charged amino acids are required for the stable cytoplasmic localization of the fused alkaline phosphatase. We propose that, in addition to hydrophobic transmembrane segments, positively charged amino acids in the hydrophilic cytoplasmic domains of a membrane protein are determinants of the protein's topology.

H 411 CONVERSION OF A CLASS II INTEGRAL MEMBRANE PROTEIN INTO A SOLUBLE AND EFFICIENTLY SECRETED PROTEIN: MULTIPLE INTRACELLULAR AND EXTRACELLULAR OLIGOMERIC AND CONFORMATIONAL FORMS. Reay G. Paterson and Robert A. Lamb. Dept. of Biochem. Molec. Biol. & Cell Biol., Northwestern University, Evanston IL 60208-3500

The N terminus of the F_1 subunit of the paramyxovirus SV5 fusion protein (fusion related external domain, FRED) is a hydrophobic domain that is implicated in mediating membrane fusion. We have examined the ability of the FRED to function as a combined signal-anchor domain by substituting it for the natural N-terminal signal-anchor domain of a model type II integral membrane protein: the influenza virus neuraminidase. The hybrid protein (NAF) was expressed in eukaryotic cells. The FRED was shown to act as a signal sequence, targeting NAF to the lumen of the endoplasmic reticulum (ER), by the fact that NAF acquired N-linked carbohydrate chains. NAF is a soluble protein in the lumen of the ER and the results of N-terminal sequence analysis showed that the FRED is cleaved at a site predicted to be recognized by signal peptidase. NAF was found to be efficiently secreted ($t_{1/2}$~90 min) from the cell. By using a combination of sedimentation velocity centrifugation and immunoprecipitation assays using polyclonal and conformation-specific monoclonal antibodies it was found that extracellular NAF consisted of a mixture of monomers, disulfide-linked dimers and tetramers. The majority of the extracellular NAF molecules were not reactive with the mAbs suggesting they were not folded in a native form and only the NAF tetramers had matured to a native conformation such that they exhibited neuraminidase activity. The available data indicates that NAF is transported intracellularly in multiple oligomeric and conformational forms.

H 412 ANALYSIS OF TOPOGENIC SIGNALS IN MEMBRANE-SPANNING PROTEINS

Hans Peter Wessels, James P. Beltzer, Iris Geffen and Martin Spiess,
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In order to study the insertion of multi-spanning membrane proteins into the endoplasmic reticulum, we constructed artificial proteins on the cDNA level by repeating up to four times the internal signal-anchor domain of the asialoglycoprotein receptor H1. Upon in vitro-translation in the presence of microsomes, the first hydrophobic domain functions as a signal and the second as a stop-transfer sequence, while the third initiates a second translocation process, halted again by the fourth. [Ref. 1]. We replaced the third transmembrane segment by a normally cleaved amino-terminal signal sequence of influenza virus hemagglutinin. Efficient cleavage was observed, demonstrating that processing by signal peptidase is not necessarily restricted to the first hydrophobic domain of nascent proteins [Ref. 2]. Twofold membrane-spanning constructs with a set of different hydrophobic domains in the second position were used to study the characteristics of stop-transfer sequences. We discovered that some behave differently depending on the translation system used. Expression in fibroblasts showed that the reticulocyte lysate system reflects more closely the in vivo situation than the wheat germ system. Recently it has been proposed that the difference in the charges of the 15 residues flanking a first internal signal-anchor determines its orientation in the membrane upon insertion [Ref. 3]. Currently we are testing this hypothesis by systematically altering the membrane-flanking charged residues in H1.

1. Wessels, H.P., and Spiess, M. (1988) Cell *55*, 61-70. 2. Beltzer, J.P. et al. (1989) FEBS Lett. *253*, 93-98. 3. Hartmann, E. et al. (1989) Proc. Natl. Acad. Sci. USA *86*, 5786-5790.

H 413 SCRAPIE AND CELLULAR PRION PROTEINS DIFFER IN THEIR KINETICS OF SYNTHESIS AND TOPOLOGY IN CULTURED CELLS, David R. Borchelt, Michael Scott, Albert Taraboulos, Neil Stahl, and Stanley B. Prusiner, University of California, San Francisco, CA 94143

Both the cellular and scrapie isoforms of the prion protein (PrP) designated PrP^C and PrP^{Sc} are encoded by a single-copy chromosomal gene and appear to be translated from the same 2.1-kb mRNA. PrP^C can be distinguished from PrP^{Sc} by limited proteolysis under conditions where PrP^C is hydrolyzed and PrP^{Sc} is resistant. We report here that PrP^C can be released from the surface of both normal-control and scrapie-infected murine neuroblastoma (N_2a) cells by phosphatidylinositol-specific phospholipase C (PIPLC) digestion and it can be selectively labeled with sulfo-NHS-biotin, a membrane-impermeant reagent. In contrast, PrP^{Sc} was neither released by PIPLC nor labeled with sulfo-NHS-biotin. Pulse-chase experiments showed that [^{35}S]-methionine was incorporated almost immediately into PrP^C while incorporation into PrP^{Sc} molecules was observed only during the chase period. While PrP^C is synthesized and degraded relatively rapidly ($t_{½}$ ~5 h), PrP^{Sc} is synthesized slowly ($t_{½}$ ~15 h) and appears to accumulate. These results are consistent with several observations previously made on rodent brains where PrP mRNA and PrP^C levels did not change throughout the course of scrapie infection, yet PrP^{Sc} accumulated to levels exceeding that of PrP^C. Our kinetic studies demonstrate that PrP^{Sc} is derived from a protease-sensitive precursor and that the acquisition of proteinase K resistance results from a post-translational event. Whether or not prolonged incubation periods which are a cardinal feature of prion diseases reflect the slow synthesis of PrP^{Sc} remains to be established.

H 414 STRUCTURAL REQUIREMENTS OF THE SIGNAL/ANCHOR DOMAIN FOR THE INTRACELLULAR TRANSPORT OF INFLUENZA NEURAMINIDASE,

Brenda G. Hogue, Raymond F. Castro, Hetal S. Patel, and Debi P. Nayak, Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90024

The influenza virus neuraminidase (NA) is a class II protein with a stretch of 29 hydrophobic amino acids (residues 7-35) at its amino-terminus that provides both the signal and the anchor functions. A series of mutations were made in this region to analyze the requirements for the signal and anchor functions. The mutants were analyzed using an in vitro transcription-translation system. It was found that not all of the hydrophobic residues are required for either the signal or the anchor functions. A functional signal for translocation into the ER could be provided by 15-16 residues at the carboxy end or 10-11 residues at the amino end of the hydrophobic domain. All translocation-positive mutants were anchored; however, anchoring stability among the proteins was variable. Some of the mutants appeared to be cleaved, most likely by signal peptidase. The role of the signal/anchor domain in the transport of NA to the cell surface was also analyzed by expression of translocation- positive mutants in CV-1 cells, using a vaccinia virus expression system. Some of the translocation-positive mutants were blocked in their transport to the cell surface. The blocked mutants had reduced enzymatic activity and appeared not to form their normal tetrameric structure. The data suggest that the signal/anchor domain is involved in oligomerization and is therefore important in transport of the protein to the surface.

H 415 INTRACELLULAR TRANSPORT OF MHC-CLASS II ANTIGENS AND ASSOCIATED INVARIANT CHAIN IN A HUMAN MELANOMA CELL LINE, Jean Pieters, Joachim Lipp and Bernhard Dobberstein, EMBL, P.O. Box 102209, Heidelberg, FRG.

Class II molecules consist of two integral membrane proteins (α and β chain) and are known to function in the processing and presentation of class II restricted antigens. This involves transport from their place of synthesis to an intracellular compartment in which antigen processing occurs. Class II molecules are associated with a third protein, the invariant chain (Ii), for which an important role in the intracellular transport of class II molecules has been suggested. We have investigated the intracellular transport of MHC class II antigens (α,β) and associated invariant chain (Ii) in a human melanoma cell line. Soon after their synthesis, class II molecules and Ii assemble in the endoplasmic reticulum. Pulse-chase studies show that the oligomeric complex (α,β,Ii) becomes glycosylated after 1 hour. In contrast to the α and β chain, which remain very stable, Ii is degraded in a time course of 4 hours. This degradation occurs stepwise, first to a 20-22 kD molecule, which is then further degraded to a 10 kD peptide. Both degradation products are derived from the N-terminus of Ii, and remain associated with the α and β chain. Our results suggest that, after transport of the oligomeric complex (α,β,Ii) to the intracellular compartment, the luminal part (C-terminus) of the Ii is degraded. This compartment is most likely a prelysosome/late endosome, as the Ii colocalises with the mannose-6-phosphate receptor in electron microscopy studies. We suggest that the continuous association of Ii or its degradation products with class II molecules may regulate the intracellular transport of class II MHC molecules to the cell surface.

H 416 **RER-LOCALISED ROTAVIRUS NS28 PROTEIN: TOPOLOGY OF THE RECEPTOR AND ITS INTERACTION WITH THE LIGAND,** A. Richard Bellamy, Janice C. Meyer, Jeanette E. Street, Indumathy Anthony, John Berriman and Cornelia C. Bergmann, Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand

The rotavirus non-structural glycoprotein, NS28, is an RER targeted integral-membrane glycoprotein that is anchored with the carboxy-terminal domain exposed on the cytoplasmic side of the membrane (Bergmann et al, EMBO J 8, 1695, 1989). The protein is uncleaved and the two glycosylation sites at positions 8 and 18 are filled with high mannose carbohydrate, indicating localisation of the protein to the ER compartment. Rotaviruses assemble in the lumen of the ER by budding from the ER membrane. The budding event is preceded by a specific interaction between the core and NS28 in which NS28 acts as the receptor and the major surface protein of the core (VP6) is the ligand. This interaction therefore provides a model for the events involved in the budding of icosahedral particles across membranes.

We have investigated the topology of NS28 in the membrane and its interaction with rotavirus cores. The aim of the work is to characterise and identify the domain(s) of the receptor involved. Engineered variants of NS28 have been delivered to the ER using both baculovirus and vaccinia virus vectors and the receptor activity measured using ^{125}I-labelled cores. The morphology of the ligand has also been investigated by taking advantage of the observation that VP6 forms two-dimensional paracrystalline arrays that are amenable to study by electron microscopy. The results of this work will be presented together with some preliminary data on the nature of the receptor:ligand complex.

H 417 INHIBITION OF THE SYNTHESIS OF GLYCOLIPID PRECURSORS OF THE MEMBRANE ANCHOR OF TRYPANOSOME VARIANT SURFACE GLYCOPROTEINS, S. Mayor, R.T. Schwarz*, A.K. Menon, and G.A.M. Cross. The Rockefeller University, New York, NY, 10021 U.S.A. and *Philipps-Universitaet Marburg, Marburg, F. R. G.

Several eukaryotic cell surface proteins, including the variant surface glycoproteins (VSGs) of *Trypanosoma brucei*, are anchored to the membrane by a glycosylinositol phospholipid (GPI). The GPI anchor is covalently linked to the carboxyl-terminus of the protein and appears to be acquired immediately upon completion of protein synthesis. Many GPI anchored proteins, including VSG can be released from the membrane by a phosphatidylinositol-specific phospholipase C (PI-PLC). However, some are resistant to this enzyme. Putative PI-PLC sensitive (P2) and resistant (P3) glycolipid precursors to the GPI-anchor have been identified and characterized in *T. brucei*. P2 and P3 contain ethanolamine-phosphate-Man_3GlcN linked glycosidically to the inositol residue, as do all GPI anchors that have been structurally characterized. The single difference between P2 and P3, and the basis of the PI-PLC insusceptibility of P3, is a fatty acid ester linked to the inositol residue in P3. 2-Fluoro-2-deoxy-D-glucose (F-Glc), a known inhibitor of Dol-P-Man synthesis, has been shown to inhibit the synthesis of P2 and P3 *in vivo* (Schwarz, R.T., Mayor, S., Menon, A.K., and Cross, G.A.M.(1989) *Biochem. Soc. Trans. Lond.* **17** 746-748). The inhibition of synthesis of P2 and P3 is accompanied by the accumulation of a biosynthetic intermediate, GlcN-PI. The data obtained support the hypothesis that Dol-P-Man is involved in the formation of the mannose core of the precursor to the VSG glycolipid anchor and is consistent with the pathway of glycolipid synthesis described *in vitro* (Menon, A.K., Schwarz, R.T., Mayor, S., and Cross, G.A.M. *J. Biol. Chem.* submitted; Masterson, W.J., Doering, T.L., Hart, G.W., and Englund, P T. (1989) *Cell* **56** 793-800). The effect of F-Glc on the synthesis and processing of VSGs is under investigation.

H 418 CELL-FREE SYNTHESIS OF GLYCOSYLPHOSPHATIDYLINOSITOL PRECURSORS FOR THE GLYCOLIPID MEMBRANE ANCHOR OF *TRYPANOSOMA BRUCEI* VARIANT SURFACE GLYCOPROTEINS A. K. Menon, R. T. Schwarz, S. Mayor, M.C. Field, and G. A. M. Cross. The Rockefeller University, New York, NY 10021. USA.
Trypanosome variant surface glycoproteins exemplify a class of eukaryotic cell-surface glycoproteins that utilize a carboxyl-terminal, covalently attached, inositol-containing glycophospholipid for membrane attachment. The glycolipid anchor is acquired rapidly upon completion of protein synthesis [Cross, *Cell* **48**, 179 (1987)] apparently by replacement of a short carboxyl-terminal peptide sequence with a pre-fabricated glycolipid. Candidate glycolipid precursors (P2 and P3) to the VSG anchor have been identified *in vivo* [Krakow *et.al.*, *J. Biol. Chem.* **261**, 12147 (1986); Menon *et.al.*, *J. Biol. Chem.* **263**, 1970 (1988)]. We have recently described a trypanosome membrane preparation capable of synthesizing P2 and P3 from endogenous substrates and GDP-[^{3}H]mannose or UDP-[^{3}H]GlcNAc [Menon *et al.*, *Biochem. Soc. Trans. Lond.* **16**, 996 (1988)]. Analyses of organic solvent extracts from labelled membranes resolved a spectrum of partially glycosylated biosynthetic intermediates consistent with a sequential glycosylation pathway for anchor assembly. Using the cell-free system, the source of all three mannose residues in the anchor structure was identified as Dol-P-Man. [^{3}H]Mannose incorporation was stimulated by the addition of Dol-P and reduced in the presence of amphomycin, an inhibitor of Dol-P-Man synthesis, whilst all three mannose residues (identified by methylation analysis) could be labelled by incubating membranes with dol-P-[^{3}H]mannose.

H 419 AN UNCLEAVED AMINO-TERMINAL SIGNAL SEQUENCE CAN FUNCTION AS A MEMBRANE ANCHOR, Robert G. Shatters, Jr. and Jan A. Miernyk, Seed Biosynthesis Research Unit, USDA, ARS, Northern Regional Research Center, Peoria, IL 61604.
To study the functions inherent to the signal sequence of the maize storage protein zein, we have constructed a gene encoding the Z4 zein signal sequence at the amino terminus of the cytosolic maize Adh protein. Translocation and processing of this chimeric protein, prezad, were assayed in vitro by using a rabbit reticulocyte translation system supplemented with canine pancreatic microsomes. As determined by protease resistence, prezad was translocated into the microsomes; however, the signal sequence was not removed. Further analysis, by alkaline carbonate extraction, showed that prezad was integrated into the microsomal membranes. Structural analysis of prezad showed two potential hydrophobic membrane spanning regions: the signal sequence and a sequence between residues 215 and 234. Translocation analysis of truncated prezad constructs implicates the uncleaved signal sequence as the membrane anchor. Protection of the entire prezad sequence from proteolysis and determination of the amino terminus as the membrane anchor indicate a type II membrane orientation for prezad. It has been previously proposed that a net positive charge within the first 16 amino acids carboxy-terminal to an uncleaved signal sequence can stabilize a type I membrane orientation. However, prezad has a net 2+ charge within the first 10 amino acids of Adh and a type II membrane orientation. We propose that the secondary structure of the border between the signal sequence and its carboxy-terminal flanking residues may be important in both signal peptidase recognition and orientation of membrane insertion.

H 420 REDISTRIBUTION OF AN ACTIN-BINDING PROTEIN FROM PERINUCLEAR TO LEADING EDGE OR GROWTH CONE ACCOMPANIES MIGRATION OR NEURITE OUTGROWTH. E.L. Bearer. Dept. of Biochemistry. Univ. of Cal. S.F. CA 94143.
The leading edge of a migrating cell is a specialized subcellular compartment composed of a dynamic actin filamentous network and devoid of organelles.This region of the locomoting cell has been shown to be enriched in a variety of actin-binding proteins as well as membrane-associated molecules, although how these molecules come to be concentrated in this region of the cell is not known. I have raised polyclonal and monoclonal antibodies to novel actin-binding proteins isolated by F-actin affinity chromatography, and found several that stain the leading edge of migrating chick embryo fibroblasts and the ruffle of the growth cone of PC-12 cells induced to differentiate. One of these antibodies, Mab 2E4, recognizes a protein of 43 kD apparent molecular weight that elutes with ATP from F-actin affinity columns loaded with ADP-activated platelet extracts.Furthermore, ATP extraction of permeabilized fibroblasts selectively removes the staining at the leading edge by Mab 2E4 while leaving the actin stress fibers intact. While Mab 2E4 stains both the perinuclear region as well as the leading edge of migrating cells, only perinucleur staining is seen in confluent cultures of fibroblasts and in undifferentiated PC-12 cells. The location of the staining suggests that the antigen is either in or near the Golgi apparatus and centrosomal region of the cell. I am currently investigating whether these organelles play a role in the redistribution of this actin-associated protein during induction of neurite outgrowth in PC-12 cells by co-localizing the Mab 2E4 antigen both biochemically and by immunofluorescence with Golgi markers, anti-centrosomal antibodies, clathrin, and p38-containing vesicles, and by observing the effect of microtubule destabilizing drugs on its delivery to the growth cone.

H 421 DISTRIBUTION OF SPECTRIN AND MONOLAYER FORMATION IN A MDCKII CELL CULTURE IS AFFECTED BY INTRACELLULAR pH, Sinikka Eskelinen, Virva Huotari, Raija Sormunen and Veli-Pekka Lehto, Biocenter and Department of Pathology, University of Oulu, SF-90220 Oulu, Finland

A rapid and reversible uncoupling of cell-cell contacts can be achieved by acidification by nigericin treatment of epithelial MDCKII cells grown in a continuous monolayer. Concomitantly, spectrin is released from its normal residence in the region of the basolateral plasma membrane and observed throughout the cytoplasm. The reorganization takes place in 10 minutes and the pH treshold is 6.0 - 6.2. The phenomenon is reversible and spectrin is reassembled at the basolateral membranes in less than 10 minutes after restoring a normal pH. Other cytoskeletal components (tubulins, vimentin and actin) remain immobile during acidification of the cells. The lack of cell-cell contacts and the diffuse distribution of spectrin in acidified cells is similar to that observed in freshly plated epithelial cells or cells grown in a low Ca^{2+} medium. The results show a close association between regulatory proton and calcium ion gradients and the organization of cell-cell contacts and the spectrin-based membrane skeleton.

H 422 "IN SITU" TRANSLATION: USE OF THE CYTOSKELETAL FRAMEWORK TO DIRECT CELL-FREE PROTEIN SYNTHESIS, Joel S. Pachter and Diane Biegel, Department of Physiology, University of Connecticut Health Center, Farmington, CT 06032

While many examples of gene regulation occurring at the level of translation are evident throughout the literature, such as alterations in either translation initiation rate, polypeptide elongation rate, translation termination rate, or the stability of translating mRNAs, as well as the segregation of particular mRNAs to specific subcellular domains, there is little data illuminating the molecular mechanisms governing these events. However, given that the majority of ribosomes engaged in translation are found associated with the cytoskeletal framework, it is intriguing to speculate that the cytoskeleton may play an active role in regulating both the extent and locale of gene expression. We have, therefore, developed a cytoskeletal-based protein synthesizing system from cultured mouse cells to aid in examining the molecular basis for such regulatory events. By using a low concentration of nonionic detergent to gently extract cells while they are still adherent to a substrate, and avoiding such destructive preparative techniques as cellular homogenization and high speed centrifugation, we have obtained a system that is devoid of membraneous barriers yet retains much the same topological arrangement of mRNA, ribosomes and cytostructure that exists "in situ". Preliminary data indicate that when this system is supported by soluble factors from a reticulocyte lysate, it is capable of continually translating endogenous, cytoskeletal-bound mRNAs for at least 40 minutes. Emulsion autoradiography of ongoing protein synthesis demonstrates that protein synthetic activity is ubiquitous throughout the population of extracted cells, and not confined to a possibly less well extracted subset. SDS-PAGE reveals that the pattern of proteins produced by such extracted cells is identical to that produced by unextracted cells, including proteins of molecular weight greater than 200Kd. Furthermore, a continued increase in intensity of almost all proteins during this time period strongly suggests that translational reinitiation is also taking place. This system should prove useful in examining the role the cytoskeleton plays in the subcellular compartmentalization of mRNA and in regulating gene expression.

H 423 **Binding To Microtubules Of A Cytoplasmically Oriented, Peripheral Membrane Protein Of The Golgi Apparatus: A Potential Mechanism For Positioning the Golgi Near The Microtubule-Organizing Center.** G.S. Bloom, T.A. Brashear and Y.-C. Lin. Dept. Cell Biol. & Neurosci., U. TX Southwestern Med. Ctr.; Dallas, TX 75235.

The Golgi Apparatus typically resides near the microtubule-organizing center (MTOC), and relies on the presence and organization of microtubules (MTs) to maintain its structural integrity and intracellular location. Accordingly, there must be factors responsible for linking the Golgi to MTs and positioning it near the MTOC. A new MT-binding protein exhibits properties consistent with its involvement in both of these processes. This M, 58,000 protein (58K) was purified from rat liver cytosol by a three step procedure: 1) binding to exogenous, taxol-stabilized MTs; 2) salt extraction of the MTs; and 3) gel filtration chromatography. Based on immunological, peptide mapping and microsequencing experiments, 58K was found to represent a novel protein species. To explore its *in vivo* function, two lines of investigation were pursued. First, several monoclonal antibodies to 58K were used to determine its subcellular distribution. Immunofluorescence of hepatoma cells yielded staining of interconnected, perinuclear tubules and stacks, which, in cells exposed to taxol or colchicine, were disrupted into particles scattered throughout the cytoplasm. The immunoreactive structures co-localized with material stained by fluorescent wheat germ agglutinin, establishing their identity as Golgi membranes. Complementary western blotting experiments revealed substantial levels of 58K to be present in Golgi membranes isolated from rat liver. Moreover, immunoreactive 58K behaved as a cytoplasmically oriented, peripheral membrane protein in these Golgi isolates following their extraction with Triton X-114 or KCl, or exposure to chymotrypsin. The second line of investigation regarding the *in vivo* function of 58K was aimed at its interactions with MTs. In purified form, 58K bound directly to MAP-free MTs, stimulated tubulin polymerization weakly and, consistent with the latter observation, saturated MTs at a 1:1 molar ratio relative to tubulin. Collectively, our data indicate that 58K is appropriately situated on the Golgi apparatus to permit binding to MTs, and suggest that it serves as an anchorage site for MTs on the Golgi surface. Furthermore, the relatively modest affinity of 58K for MTs might passively cause the Golgi to become situated where the number of weak, 58K-mediated interactions with MTs could be maximized. This location would likely be centered around the MTOC, where MTs are most densely packed in the cell.

H 424 SAC1p, AN INTEGRAL MEMBRANE PROTEIN THAT MODULATES YEAST GOLGI AND YEAST ACTIN FUNCTION, Ann E. Cleves and Vytas A. Bankaitis, Department of Microbiology, University of Illinois, Urbana, IL 61801. We have shown that the SAC1 gene product of S. cerevisiae significantly influences the states of both the yeast secretory pathway (at the level of Golgi function) and the yeast actin cytoskeleton. As a result, we investigated the distribution of Sac1p in the yeast cell. Using a TrpE-Sac1 fusion protein, we have generated monoclonal antibodies to Sac1p. Sac1p is an unglycosylated polypeptide with an apparent molecular weight of 65Kd. Subcellular fractionation experiments have indicated that Sac1p is found exclusively in the 12,000xg and 100,000xg pellet fractions. Evidence indicating Sac1p to be an integral membrane protein is obtained by our observation that Sac1p is not efficiently extracted from membranes by treatment with Na_2CO_3, pH 11.5. Hydropathy analysis of the inferred Sac1p amino acid sequence indicates that Sac1p does exhibit an excellent candidate for a potential membrane-spanning domain. Preliminary results of protease protection experiments, employing cell lysates in which the integrity of small organelles is maintained, show that most of Sac1p is resistant to digestion by proteinase K in the absence of detergent, while Sac1p is completely digested in the presence of detergent. This result suggests that Sac1p may have both a cytoplasmic and a lumenal domain.

H 425 IDENTIFICATION OF A NUCLEAR TARGETING SIGNAL IN A BACULOVIRUS-ENCODED PROTEIN AND BACULOVIRUS-MEDIATED ENHANCEMENT OF NUCLEAR PROTEIN LOCALIZATION. Donald L. Jarvis. Department of Entomology; Texas A&M University, College Station, TX 77843. Nuclear targeting signals have been identified in a number of proteins encoded by mammalian viruses. These usually consist of several basic amino acids, often flanked by proline residues. Nuclear targeting signals in some yeast proteins are different, with a core of three hydrophobic amino acids flanked on both sides by basic amino acids. We have identified a nuclear targeting signal in a baculovirus-encoded protein, polyhedrin, which accumulates in huge amounts in the nuclei of infected insect cells. Increasing lengths of the polyhedrin coding sequence were fused in-frame to a sequence encoding the cytoplasmic protein, ß-galactosidase (ß-gal). Each hybrid gene was engineered into a recombinant baculovirus and expressed in infected insect cells. The subcellular distribution of the fusion products was determined by indirect immunofluorescence with anti-ß-gal as the probe. Fusion proteins containing polyhedrin amino acids 1 to 11, 22, 25 or 28 were cytoplasmic, while those containing amino acids 1 to 30, 57 or 110 were nuclear. These observations were extended by biochemical fractionation of the infected cells followed by immunoprecipitation or western analysis of the fusion products. The results confirmed that a minimal nuclear targeting signal is located within the first 30 amino acids of polyhedrin. The sequence of basic amino acids (KRKKH) from positions 32 to 36 is not an essential part of this signal. Instead, polyhedrin, which normally is expressed in insect cells, appears to contain a yeast-like nuclear targeting signal. Quantitative analysis of the intracellular distribution of various fusion products at early or late times of infection revealed that those which contained the nuclear targeting signal were more exclusively nuclear at later times of infection. This was not a nonspecific effect of the viral infection on the nuclear envelope, as cytoplasmic constructs remained strictly cytoplasmic. We hypothesize that this baculovirus encodes or induces a function that enhances nuclear protein localization during the later times of infection.

H 426 THE HYDROPHOBIC C-TERMINUS OF THE MUTANT SV40 T ANTIGEN 676FS INTERFERES WITH NUCLEAR TRANSPORT, Karen Lister van Zee, Frank Appel, Erika Schreder, Ellen Fanning, Institute for Biochemistry, Ludwig-Maximilian Univeristy, Munich, West Germany. A hydrophobic domain present at the C-terminus of the SV40 T-antigen mutant 676FS interferes with the nuclear transport of this protein and some but not all heterologous proteins (1). The nuclear Adenovirus 5 fiber and 72kd DNA binding proteins show a significant cytoplasmic accumulation in the presence of the FS T antigen; however, the localization of Adenovirus E1A is not detectably altered. The hydrophobic carboxy terminus of the 676FS is also able to inhibit nuclear transport in different protein contexts. ß-galactosidase fusion proteins bearing an SV40 T antigen nuclear location signal and the 676FS C-terminus resemble 676FS in their subcellular distribution. However, the fusion proteins do not interfere detectably with the transport of heterologous proteins.

A similar hydrophobic region located at the carboxy terminus of the membrane associated polyoma middle T is also able to prevent the nuclear accumulation of a middle T antigen bearing the SV40 T antigen nuclear location signal (2). Based on this observation and the results described above, the FS hydrophobic C-terminus may anchor the protein in the cytoplasm. Unable to reach the nucleus, the FS protein could sequester a cytoplasmic factor whose role is to recognize a nuclear location signal and shuttle proteins bearing such a signal to the nucleus. If produced in sufficient quantity, the FS protein could titrate out this factor(s) which is presumably required for heterologous protein transport. To test this model, an analysis of 676FS bearing a polyoma middle T antigen sequence in place of its own hydrophobic sequence is under way. In addition, the kinetics of nuclear uptake as a function of 676FS and heterologous protein concentration will be tested in microinjection assays.

(1) Schneider et al. 1988. Cell 54, 117-125. (2) Roberts et al. 1987. Cell 50, 465-475.

H 427 ISOLATION OF A DOMINANT SUPPRESSOR OF A NUCLEAR LOCALIZATION SIGNAL DEFECT IN *S.cerevisiae*, Sibylle Feucht, Verena Schlup and Michael N. Hall, Dept. of Biochemistry, Biozentrum, University of Basel, Basel, Switzerland
We have taken a genetic approach to elucidate the molecular mechanism by which specific proteins are imported into the nucleus of the yeast *S. cerevisiae*. Mutation of the amino-terminal nuclear localization signal of the mating-type regulatory protein α2, product of the *MATα2* gene, confers a sterile phenotype. Eighteen mutants in which the nuclear localization signal defect is suppressed were isolated by screening for restoration of mating ability. The suppressor mutations i) are all second-site mutations in a single nuclear gene which we have called *SUN1*, ii) suppress only those mutations in α2 which affect the amino-terminal localization signal, and iii) are dominant. These properties are consistent with the possibility that *SUN1* encodes the component of the nuclear import machinery with which the defective localization signal in α2 normally interacts. A *SUN1*$^-$ allele has been cloned and is currently being characterized.

H 428 IN VITRO NUCLEAR PROTEIN LOCALIZATION IN Saccharomyces cerevisiae, Jose F. Garcia-Bustos, Philipp Wagner and Michael N. Hall, Department of Biochemistry, Biocenter of Basel University, 4056 Basel, Switzerland. We are developing a homologous in vitro system to analyze the mechanism by which proteins are localized to the yeast nucleus. The yeast transcriptional regulator Mcm1 was used as the substrate for import into purified yeast nuclei. Using this system we have observed nuclear localization of Mcm1 and other yeast nuclear proteins. Non-nuclear proteins are not localized to the nucleus under the same conditions. Mcm1 associates with nuclei in a time- and temperature-dependent manner. The association requires hydrolysis of nucleotide triphosphates, since it is supported by ATP and (partially) by GTP but not by a non-hydrolyzable ATP analog.

H 429 DOMAINS OF NUCLEAR LAMINS INVOLVED IN THEIR TARGETING TO THE NUCLEAR ENVELOPE. Thomas Höger and Georg Krohne, Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany.
We have analyzed domains in lamin molecules which are responsible for their correct targeting to and association with a pre-existing lamina in vivo and in vitro. Our in vivo results were obtained by microinjecting radioactively labelled soluble lamin molecules into the cytoplasm of Xenopus oocytes. By these means we have demonstrated that after transport of molecules into the nucleus the cysteine of the highly conserved carboxy-terminal tetrapeptide CXXM is in B-type lamins required for a stable association with the nuclear lamina. Moreover, addition of this tetrapeptide to human lamin C, which in our experimental system remains soluble in the nucleoplasm, leads to a stable association with the nuclear envelope (NE). Deletion of other parts of lamin molecules indicate that the CXXM domain is insufficient for maintenance of a stable association with the NE but that this domain is required for initiating this process. In vitro analysis of this process using isolated nuclei and nuclear envelopes confirm our in vivo results. We are further investigating the role of non-lamin components during lamin association with the NE by differential extraction of the NE prior to incubation with in vitro synthesized soluble lamin molecules.

H 430 CHARACTERIZATION OF TWO NUCLEAR LOCALIZATION SEQUENCE-BINDING PROTEINS IN RAT LIVER NUCLEI, U. Thomas Meier and Gunter Blobel, Rockefeller University, New York, NY 10021.

The nuclear import of large proteins is mediated by specific nuclear localization sequences (NLS) within these proteins. The best characterized NLS is the one of SV40 large T antigen. A single mutation in this NLS abolishes nuclear import completely in vivo and in vitro. We employed wild type (wt) and mutant (mt) synthetic NLS peptides crosslinked to human serum albumin (peptide conjugates) to test for their binding to proteins of rat liver nuclei on Western Blots. Proteins of 140kd and 55kd were specifically recognized by wt peptide conjugates. The binding to these proteins could be competed for by free wt but not mt peptides. In cell fractionation experiments these proteins were exclusively localized to the nucleus and were extractable from nuclei by either repetitive no salt extractions or high salt extraction. The two proteins may be identical to p140 and p55 identified by photoaffinity labelling with NLS peptides by L. Yamasaki et al. Mol. Cell Biol. 9, 3028 (1989). Polyclonal antibodies raised against the partially purified proteins excised from polyacrylamide gels recognize both proteins on Western Blots and give a distinct punctate nucleolar staining in immunofluorescence on permeabilized BRL cells. Purified IgG's reactive with p140 do not interfere with nuclear protein import in an in vitro import assay system. Moreover, fluorescently labeled IgG's are excluded from nuclei in the same system. Further studies will reveal how these specific NLS-binding proteins are involved in nucleocytoplasmic transport or what other function they exhibit.

H 431 PRODUCTION OF ANTIBODIES TO SYNTHETIC PEPTIDES USING PEPTIDE-PHOSPHOLIPID COMPLEXES. Raphael J. Mannino, Leslie E. Eisele, and Gail Goodman-Snitkoff. Department of Microbiology & Immunology, Albany Medical College, Albany, NY 12208

The capacity to raise high titer antisera and monoclonal antibodies to synthetic peptides representing regions of biologically interesting proteins is a powerful tool in contemporary cell biology. However, raising antibodies to synthetic peptides, which define small portions of a protein of interest, has been tedious at best. Current technologies which involved coupling a peptide to a carrier protein and immunizing in the presence of adjuvants, such as Freund's, result in the production of antibodies directed primarily to the carrier and the adjuvant with little antibody produced to the peptide of interest. An alternative approach has been developed in our laboratory. In attempting to determine minimal essential components required to construct a well defined immunogenic composite, we have found that when synthetic peptides are provided with a hydrophobic tail through crosslinking to a phospholipid and then assembled into a peptide-phospholipid composite the peptides are immunogenic. An immunogenic mixture is comprised of two essential components: 1. Each composite must contain peptides representing both B-cell determinants and T-helper (Th) determinants presented either contiguously, i.e. part of the same synthetic peptide, or on individual peptides, or trace amounts of a detergent extract of the envelope of Sendai virus to act as a "generic" provider of Th-cell determinants can be included in the composites. 2. To be immunogenic these peptides must be covalently coupled to phospholipid and inoculated as a peptide-phospholipid conjugate. No other carriers or adjuvants are required to induce an immune response. Since the peptide is the primary antigen in the composite (30-50ug), a substantial portion of the antibody produced following immunization is directed toward the peptide, even when Sendai virus envelope proteins (1ug) are included as a "generic" provider of T cell help. The antipeptide antibodies produced cross react with the native protein from which the peptide sequences were derived. The technology reported here allows a significant enhancement of antibody production to a normally poorly immunogenic moiety. This enhancement should streamline the purification of proteins.

H 432 **COMPARTMENTALIZATION OF PHOSPHOLIPIDS FOR LIPOPROTEIN SECRETION, Jean E. Vance, Lipid and Lipoprotein Group and Department of Medicine, University of Alberta, Edmonton, Ab. T6G 2S2, CANADA.**

Phospholipids utilized for assembly into lipoproteins by cultured rat hepatocytes are selected from specific pools according to their routes of biosynthesis [Vance, J.E. and Vance D.E. (1986) J. Biol. Chem. 261, 4486-4491]. In addition, newly-synthesized, rather than pre-existing, phospholipids are preferred for secretion. Surprisingly, one of the pools of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) preferred for secretion is that derived from decarboxylation of phosphatidylserine (PtdSer) to PtdEtn using a reaction that occurs exclusively in mitochondria. How does such a pool of mitochondrial phospholipid remain distinct from the bulk of phospholipid in the ER or Golgi during lipoprotein assembly? A rat liver membrane fraction with the following properties has been recently isolated. (i) It sediments with mitochondria, not with microsomes. (ii) When separated from mitochondria on a Percoll gradient the membrane contains low marker enzyme activity for ER (NADPH:cytochrome c reductase), mitochondria (cytochrome oxidase) and other organelles. (iii) The membrane fraction contains most of the enzyme activities of phospholipid biosynthesis that are normally present in the ER, except that the specific activity of PtdSer synthase is twice as high as that in microsomes and CTP:phosphocholine cytidylyltransferase activity is absent. (iv) The RNA content is only one third that of rough ER. (v) SDS-PAGE suggests that the protein profile of this membrane fraction resembles that of the ER. (vi) Immunoprecipitation demonstrated the presence of apoproteins B and E in this membrane fraction. In its originally-isolated form (i.e. in association with mitochondria) the membrane fraction is capable of synthesis of [^{3}H]PtdEtn and [^{3}H]PtdCho starting from [3-^{3}H]serine. However, when the membrane fraction is mixed with purified mitochondria the formation of [^{3}H]PtdEtn and [^{3}H]PtdCho is greatly reduced. Thus, this membrane fraction, and its associated mitochondria, may be involved in the linked synthesis of a pool of some of the phospholipids used for lipoprotein assembly. Supported by the Medical Research Council of Canada.

H 433 THE EFFLUX OF LYSOSOMAL CHOLESTEROL FROM CELLS, W.J. Johnson, A. van Tol*, G.K. Chacko, M.C. Phillips, and G.H. Rothblat, The Medical College of Pennsylvania, Philadelphia, PA, and *Erasmus University, Rotterdam, The Netherlands.

To gain insight into the transport of sterol from lysosomes to the plasma membrane, we studied the release of lysosomal cholesterol from intact Fu5AH rat hepatoma cells to high density lipoprotein (HDL) and other extracellular acceptors of plasma membrane sterol. To initiate the labeling of lysosomes, cells were incubated at 15°C with low density lipoprotein (LDL) containing [3H]cholesteryl oleate ([3H]CO). At 15°, LDL was taken up by cells but not delivered to lysosomes, preventing hydrolysis of the [3H]CO. The LDL labeling medium was removed, cells were rinsed and then warmed to 37°C in the presence of an extracellular sterol acceptor. At 37°, rapid chloroquine-sensitive hydrolysis of [3H]CO began after 10-20 min, and rapid efflux of the resulting [3H]cholesterol began after 60 min, suggesting a delay of 40-50 min between the generation of cholesterol in lysosomes and the presence of the sterol in the plasma membrane. This delay was the same (1) with a variety of sterol acceptors (HDL, phospholipid vesicles, and immunopurifed subfractions of HDL), (2) when cells were pre-enriched with cholesterol, (3) when cellular acyl CoA:cholesterol acyltransferase (ACAT) was stimulated with exogenous oleic acid. We conclude that in the Fu5AH cell, the transport of cholesterol from lysosomes to the plasma membrane requires a maximum of 40-50 min, and is unregulated to the extent that it is insensitive to the type of sterol acceptor in the medium and to the enrichment of cells with sterol. The lack of effect of increased ACAT activity implies that during this transport, little, if any, of lysosomally derived sterol passes through the rough endoplasmic reticulum, the site of ACAT.

H 434 HIGH LEVEL SECRETION OF HUMAN SERUM ALBUMIN (HSA) FROM pKD1-BASED SECRETION VECTORS USING KLUYVEROMYCES YEASTS, Reinhard Fleer, Patrice Yeh, Jérôme Bécquart, Nacima Faulconnier, Isabelle Maury, Hiroshi Fukuhara*, and Jean-François Mayaux, Biotechnology Department, Rhône Poulenc Santé, BP 14, 94403 Vitry Cedex and *Institut Curie, Centre Universitaire, 91405 Orsay, France.

Using multi-copy secretion vectors based on a cryptic plasmid (pKD1) initially isolated from K. drosophilarum (Chen X.J. et al., Nucl. Acids Res. 14 (1986) 4471-4481) we expressed a cDNA encoding human serum albumin (HSA) under control of natural or hybrid yeast promoters in several species of the yeast Kluyveromyces. High level extracellular secretion of correctly processed HSA could be obtained with several strains of K. lactis. Analysis of the N-terminal sequence of secreted HSA revealed equally efficient removal of the signal sequence, regardless whether a human or a yeast signal was used. Furthermore, the level of HSA secreted to the supernatant was identical with either signal. Detailed physico-chemical characterization of the secreted recombinant HSA (implying isoelectric focusing, native gel electrophoresis, ion-exchange chromatography, reverse-phase chromatography, tryptophan emission spectrometry, thermostability, epitope recognition by monoclonal antibodies, mass spectometry...) clearly demonstrates that the yeast derived HSA is indistinguishable from natural human HSA. The mitotic stability of albumin expressing pKD1 derivatives is species and strain dependent and is modified by promoter strength and culture conditions. Up to 90 % of HSA secreting cells were found to maintain the expression vector after 40 generations of non-selective growth. In addition, pKD1 based constructions yielded at least twenty-fold higher extracellular HSA levels then those obtained with isogenic expression cassettes integrated into different loci of the Kluyveromyces genome.