RECEPTOR-MODULATED SYSTEMS

Organizers: Michael Czech and William Catterall

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Plenary Sessions Page

Poster Sessions

Receptor-Modulated Transport Systems

Molecular Genetics of Bacterial Transporters

N 001 STRUCTURE AND REGULATION OF POTASSIUM TRANSPORT SYSTEMS IN Escherichia coli,

Wolfgang Epstein, Mark O. Walderhaug, Donald C. Dosch, James W. Polarek and Jean M. Daniel, Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637

Kdp, a three-subunit P-type K^+-ATPase, is one of at least four independent systems for the uptake of K^+ in E. coli. Its largest subunit, KdpB of 72 kDa, is acylphosphorylated and is homologous to other ATPases. The 59 kDa KdpA subunit is very hydrophobic, has at least 10 membrane-spanning regions and has no known homolog. Almost all mutants with reduced affinity for K^+ alter the KdpA subunit, such changes being clustered in 4 small regions uniformly spaced in the subunit and all predicted to be on the same side of the membrane. These results suggest that the K^+ binding site in Kdp, and at least part and perhaps all of the transmembrane channel for ion movement are formed by KdpA. If true, this implies that KdpB does not have a major role in ion binding and translocation, in contradistinction to single subunit ATPases where these functions are performed by the same subunit that couples transport to ATP hydrolysis. This implication is supported by unique features of the two pairs of closely-spaced membrane-spanning regions in the N-terminal half of KdpB (1). While the homologous pairs of such regions in other ATPases are separated by small hydrophilic stretches, as expected if the pairs extended through the membrane to the external surface, those in Kdp are not separated by hydrophilic residues. Therefore, these membrane-spanning regions of Kdp may not extend into the external medium. This difference suggests that those pairs are important in forming the transmembrane ion channel in the other ATPases, but not in Kdp where KdpA performs ion translocation.

The activity of the Kdp system, and of the other K^+ uptake systems examined, is controlled by turgor pressure, the osmotic pressure difference across the cell membrane. In the steady-state Kdp mediates exchange of K^+ but not net uptake; a reduction in turgor stimulates uptake without altering efflux so that net uptake results. The mechanism of this control, analogous to feedback control of enzymes, is not known. The expression of Kdp is under unique control: low turgor is required to express Kdp. The 98 kDa membrane bound KdpD and the 26 kDa soluble KdpE proteins are necessary for physiological expression of Kdp. The sequences of these proteins show extensive homology to the sensor-effector class of regulators, such as those controlling nitrogen assimilation, the phosphate regulon control, porins, and chemotaxis. Since phosphorylation has been shown to mediate control of several such regulators, it is suggested that KdpD serves to monitor turgor and to phosphorylate KdpE when turgor is low to stimulate expression of the three structural genes of Kdp. (Supported by NIH GM22323)

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N 002 BACTERIAL ANCESTORS OF MAMMALIAN GLUCOSE TRANSPORT PROTEINS

Peter J.F. Henderson, Paul E. Roberts, Michael T. Cairns, Stephen Baldwin*, Terence P. McDonald and Valerie A. Lucas, Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, UK.

The galactose/H^+ membrane transport protein (GalP) of *Escherichia coli* has a substrate specificity very similar to the family of three homologous glucose transporters typically found in either brain, or liver, or insulin-dependent tissues (heart, adipose tissue) of mammals, including man (1-5). By mapping, cloning and sequencing the *galP* gene, we have determined the amino acid sequence of GalP, which is similar to the sequences of the mammalian glucose transporters with 28-33% identical residues and 10-15% additional conservative substitutions. This widespread family of homologous proteins now includes the following: glucose transporters from a Cyanobacterium (6), yeast (7) and *Leishmania* (8); arabinose (AraE) and xylose (XylE) transporters from *E. coli* (9,10); and galactose, lactose and maltose transporters from yeasts (11,12). By aligning these proteins on the basis that they all contain 12 predicted membrane-spanning alpha-helices and a central hydrophilic region, we have located about 40 residues (out of 471-522) that are highly conserved and hence essential for the maintenance of structure and function. These conserved residues comprise discrete sets of sequence motifs, some of which are found at corresponding positions in other bacterial proteins for transport of citrate or tetracycline. The GalP protein is about 70% identical to AraE. The structural similarity of these two *E. coli* proteins to each other and to the mammalian glucose transporters is confirmed by their substrate-protectable reaction with [^{3}H]-cytochalasin B (K_d 0.7-2.3μM). Also, they all yield similar [^{3}H]-cytochalasin-labelled polypeptides when digested with proteases. A unifying model of the structures of these homologous sugar transporters will be discussed.

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N 003 MOLECULAR BIOLOGY OF ACTIVE TRANSPORT: MEMBRANE TO MOLECULE TO MECHANISM, H.R. Kaback, Howard Hughes Medical Institute/ University of California, Los Angeles, Molecular Biology Institute, Los Angeles, CA 90024-1570.

The *lac* permease of *E. coli* is a prototype secondary transport protein, catalyzing symport of a single β-galactoside with a single H^+. The permease is a hydrophobic transmembrane protein, encoded by the *lacY* gene, that has been solubilized, purified to homogeneity, reconstituted and shown to be completely functional as a monomer. Spectroscopic techniques demonstrate that purified permease is about 80% helical, and hydropathy profiling suggests a secondary structure in which the polypeptide consists of 12 hydrophobic segments in α-helical conformation that traverse the membrane in zig-zag fashion connected by shorter, hydrophilic domains. Support for general aspects of the model has been obtained from the proteolysis and chemical labeling and from immunological studies. Most recently, *lacY-phoA* fusion analyses have provided strong support for more specific topological predictions of the 12-helix model (J. Calamia & C. Manoil, in preparation). Site-directed mutagenesis is being utilized to probe the structure and function of the permease. Application of the technique suggests that Arg302 (helix IX), His322 (helix X) and Glu325 (helix X) may be sufficiently close to form an H-bond network that is important for substrate binding and lactose-coupled H^+ translocation.

N 004 MOLECULAR GENETICS OF LEUCINE TRANSPORT IN *E. coli*, Dale L. Oxender, Mark D. Adams, Lois M. Wagner, and Thomas J. Graddis, Dept. of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109.

The genes encoding the high affinity branched-chain amino acid transport system LIV-I have been cloned and completely sequenced. Seven genes are present on the 7568 bp DNA fragment, six of which are involved in transport. The two periplasmic binding proteins are encoded by the *livJ* (LIV-BP) and *livK* (LS-BP) genes. *livK* is the first gene in a polycistronic message that includes four genes encoding membrane components, *livHMGF*. The LIV–I genes are organized in a regulon containing two binding proteins, two hydrophobic components, and two putative ATP-binding components. Mutants have been isolated corresponding to the *livJ*, *livK*, *livH*, and *livG* genes. The *livM* and *livF* genes, for which no previous genetic evidence had been obtained, are shown also to be required for high affinity leucine transport by phenotypic complementation of a deletion strain lacking expression of all the LIV-I genes. The LivH and LivM proteins are predicted (from the nucleotide sequence) to be hydrophobic proteins containing multiple membrane spanning segments. The LivG and LivF proteins are more hydrophilic, but are tightly associated with the membrane. High expression of the hydrophobic components from a multicopy plasmid requires the presence of the additional membrane components. The four membrane subunits appear to form a complex that can be solubilized and isolated from the membrane. The LivH, LivM, LivG, and LivF proteins have been isolated by differential transfer to PVDF membranes. The starting position of each reading frame has been confirmed by amino-terminal sequencing of the purified protein and antibodies to the LivH, LivF, and LivG proteins have been raised in rabbits. Both LivG and LivF contain the consensus sequence for adenine nucleotide binding observed in other members of the transport protein superfamily. The LivF and LivG proteins exhibit primary sequence homology to the product of the cystic fibrosis gene, CFTR. In addition, the overall domain structure of CFTR is similar to the genetic organization of the LIV-I membrane subunit genes. Supported by NIH Grant GM11024.

Receptor-Modulated Transport Systems

Ion Transport ATPases

N 005 THE CALCIUM PUMPING ATPase OF THE PLASMA MEMBRANE. STRUCTURE, FUNCTION, AND REGULATION, E. Carafoli, Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zürich, Switzerland.

The plasma membrane Ca ATPase is the largest of the P-type ion pumps. Its primary structure has been established by protein chemistry and DNA cloning techniques in rat brain and teratoma cells. The deduced structure of three pump isoforms has been described in rat brain and several human isoforms have been identified by combined chemical sequencing and DNA cloning work on erythrocytes, teratoma cells, and other human cells. The human teratoma pump contains 1220 amino acids, corresponding to a M_r of 134,683. Asp 475 forms the acyl phosphate during the reaction cycle, and Lys 601 binds the ATP antagonist FITC. The calmodulin (CaM) binding domain has been identified next to the C-terminus (residues 1100-1127) using a bifunctional, cleavable, photoactivatable cross -linker: the domain resembles the CaM binding regions of other CaM-modulated enzymes, particularly in its strongly basic character and its propensity to form an amphiphilic helix. The endogenous Ca dependent protease calpain attacks the calmodulin binding domain, removing it from the pump in two steps. The translated sequence of the ATPase contains at the N- and C-side of the CaM binding domain sequences very rich in Asp and Glu which may play a role in regulating the interaction of Ca and CaM with the pump. Most likely, the high affinity Ca binding site of the catalytic cycle is somewhere else in the pump structure since the acidic stretches at the two sides of the CaM binding domain can be removed by trypsin without impairing the high affinity interaction with Ca and its transport. Work with synthetic peptides has shown that a tryptophan located in the N-terminal portion of the calmodulin binding domain may be important in the interaction of the domain with calmodulin. It has also shown that one calmodulin could bind two calmodulin binding domains, opening the possibility of pump dimerization induced by calmodulin. Ten hydrophobic domains, presumably spanning the membrane, have been tentatively identified; 4 in the N-terminal portion of the pump, 6 in the C-terminal portion. The mid portion of the pump(about 500 residues) contains no apolar transmembrane stretches. Ser 1178, located on the C-terminal side of the CaM binding domain, is phosphorylated by the cAMP-dependent kinase in the human teratoma pump isoform. This isoform is one of the two isoforms (the minor one) expressed in human red cells. Phosphorylation increases the Ca-affinity of the pump. Most of the pump isoforms differ in the C-terminal portion which contains the regulatory domains (the calmodulin binding domain and the cAMP-dependent phosphorylation site). The isoforms could thus display different regulation properties.

N 006 CALCIUM AND ATP BINDING SITES IN THE Ca-ATPase OF SARCOPLASMIC RETICULUM. David H. MacLennan, David M. Clarke, Tip W. Loo and Kei Maruyama. Banting and Best Dept. of Medical Research, University of Toronto, Toronto, Ontario M5G 1L6 CANADA

We have expressed the calcium-ATPase of rabbit fast-twitch skeletal muscle in monkey kidney (COS-1) cells, allowing us to measure calcium uptake in microsomal fractions from transfected cells. We have used site-directed mutagenesis to assay for functional consequences of alterations of specific amino acids.

Mutation of 6 residues in the transmembrane domain, each containing oxygen ligands, led to loss of calcium transport and of phosphorylation by ATP in the presence of calcium, but retention of phosphorylation by Pi in the presence and absence of calcium. This phenotype is fully consistent with loss of high affinity calcium binding sites and we conclude that Glu309, Glu771, Asn796, Thr799, Asp800, and Glu908 in transmembrane sequences 4,5,6, and 8 all contribute to calcium binding sites. Mutation of amino acids around the phosphorylation site (Asp351) presents a spectrum of phenotypes. Asp351 and Lys352 cannot be mutated without loss of function, but conservative mutations in other, surrounding residues permits at least partial function. Residues in other regions predicted to be involved in ATP binding have also been mutated in an attempt to map the topology of the ATP binding site.

Our studies suggest that ATP is bound to residues on the surface of the headpiece domain, while calcium is bound within the transmembrane domain. Transduction between these domains must involve conformational changes in elements of the headpiece, talk and basepiece leading to calcium transport. We suggest that the calcium binding sites are exposed to the cytoplasm and have high affinity in the E1 conformation. In the conversion to the E2 conformation, driven through the energy of ATP hydrolysis, the calcium binding sites lose access to the cytoplasm and gain access to the lumen and their affinity for calcium is lowered by several orders of magnitude. Thus calcium transport occurs through conformationally-induced changes in the access of calcium to the calcium binding sites and in the affinity of the calcium binding sites for calcium.

N 007 SODIUM, POTASSIUM-ATPase: CURRENT VIEW ON STRUCTURAL ORGANIZATION, Nikolai N. Modyanov, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, 117871 Moscow, USSR

Unlike other ion-transporting ATPases of the E1E2-type, the Na,K-ATPase molecule consists of two polypeptides: catalytic α-subunit and β-subunit with still unclear functions.

Different approaches were exploited for probing the protein folding in the membrane. Sequencing of products of two-stage limited trypsinolysis of the membrane-bound enzyme identified real sizes of main exposed loops and implied the α- and β-subunits to form seven and one transmembrane rods, respectively. The N- and C-termini of both polypeptides are similarly oriented relative to the plasma membrane.

The proposed disposition of the loops linking membrane segments V, VI, VII and the C-terminus of the α-subunit were confirmed by means of antibodies using immunofluorescence staining of viable cells or smears of the cell line. Raman spectra of native Na,K-ATPase and membrane-bound products of its two-stage trypsinolysis were measured to calculate the secondary structure of hydrophilic and hydrophobic regions of the enzyme. The data demonstrated that the membrane part is in the α-helical conformation.

The mutual disposition of intramembrane rods in Na,K-ATPase was studied by TID photolabeling. Today the modified fragment of the β-subunit was isolated and sequenced. The labeled residues are found predominantly on one side of the helix. The study of the α-subunit intramembrane fragments is in progress. An alkylating ATP analog was used to analyze the catalytic site topography. Target residues of affinity modification were shown to be different in E1 and E2 conformations (Asp 710 and Asp 714, respectively).

The above experimental data underlie a detailed model of the Na,K-ATPase transmembrane organization. Determination of the sequence of human genes for the enzyme subunits allowed tracing the probable correlation between the exon-intron organization of genes and structure-functional features of proteins.

N 008 THE YEAST PLASMA-MEMBRANE [H^+]-ATPase: DEVELOPMENT OF AN EXPRESSION SYSTEM FOR MUTATIONAL ANALYSIS. Robert K. Nakamoto, Rajini Rao, and Carolyn W. Slayman, Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510.

With the cloning of the PMA1 gene encoding the major proton-translocating ATPase of the yeast plasma membrane, site-directed mutagenesis has become an attractive method for the study of structure-function relationships. An essential requirement is a gene expression system where the mutant ATPase can be studied in the absence of background wild-type activity, and where ATP-driven proton transport can be assayed in addition to ATP hydrolysis. We report that *de novo* synthesized ATPase in the secretory vesicles of a temperature-sensitive *sec-6* strain can provide such a system.

The ATPase is fully mature in its catalytic and transport properties, and the vesicles are tightly enough sealed to permit the measurement of pH gradient and membrane potential with fluorescent probes. The chromosomal (wild-type) PMA1 gene is placed under galactose control so that it can be turned off at will, while in a plasma-borne (mutant) gene under heat-shock control is turned on; the secretory vesicles then contain only the mutant ATPase, which can be characterized in detail.

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Ligand-Gated Ion Channels

N 009 MOLECULAR BIOLOGY OF INHIBITORY GLYCINE RECEPTORS, Heinrich Betz, ZMBH, Universität Heidelberg, Im Neuenheimer Feld 282, 6900 Heidelberg, FRG

The postsynaptic glycine receptor (GlyR) is a chloride channel protein which mediates inhibition in spinal cord and other regions of the vertebrate central nervous system (1). Using biochemical and cDNA cloning techniques we have recently shown that this receptor is a typical member of the ligand-gated ion channel family whose subunits share amino acid sequence homology and predicted transmembrane topology with nicotinic acetylcholine and $GABA_A$ receptor proteins (2). Expression of cloned ligand-binding subunits of the GlyR in Xenopus oocytes (3) or mammalian cell lines (4) leads to formation of glycine-gated strychnine-sensitive chloride channels which resemble the receptors detected in primary spinal cord neurons. During development, different isoforms of the GlyR are detected which differ in antagonist affinity, immunological properties and primary structure of their ligand-binding subunits (5). Evidence will be presented that the motor deficiency of the mouse mutant "spastic" results from inefficient expression of the adult GlyR isoform.

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N 010 THE GLUTAMATE RECEPTOR GENE FAMILY

Heinemann, S., Bettler, B., Boulter,J., Deneris, E., Duvoisin,R., Hartley, M., Hermans-Borgmeyer, I., Hollmann, M., O' Shea-Greenfield, A., Papke, R. and Rogers, S. Molecular Neurobiology Laboratory, The Salk Institute P.O. Box 85800 San Diego, California 92138.

The glutamate receptor system is thought to be involved in the first steps of learning and memory acquisition and is perhaps the most important excitatory receptor system in the mammalian brain. We have used an expression cloning approach to identify a family of glutamate receptor genes. One gene that we have called GluR K1 codes for a functional glutamate receptor of the kainate subtype. The primary structure and the physiology of the GluR K1 glutamate receptor indicates that it is a member of the ligand-gated channel family, Hollmann,M., O' Shea-Greenfield, A., Rogers, S.W. and Heinemann, S., Cloning by functional expression of a member of the glutamate receptor family. Nature **342** 643-648 (1989).

Low stringency hybridization screening of brain cDNA libraries has identified four additional genes that code for proteins with sequence homology to the GluR K1 glutamate receptor. Two of these genes code for functional glutamate receptors. The expression of these five glutamate receptor genes has been mapped in the brain by in situ hybridization. The results suggest that the genes code for different glutamate receptors that are expressed in specific brain regions.

N 011 FUNCTIONAL SITES AND FOLDING OF THE NICOTINIC ACETYLCHOLINE RECEPTOR, Arthur Karlin, Amitabh Chak, Cynthia Czajkowski, Mario DiPaola, Donna Mielke, Eugenia Silva-Herzog and David Stauffer, Center for Molecular Recogition and Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032

The following issues will be discussed: The identification of residues at the acetylcholine binding sites; the identification of residues at noncompetitive binding sites and the implications for gating and cation-conduction; the folding of the subunits in the membrane; and the arrangement of the subunits in the three-dimensional structure of the receptor.

N 012 STRUCTURE-FUNCTION STUDIES ON MEMBRANE CHANNELS USING SITE-DIRECTED MUTAGENESIS AND HETEROLOGOUS EXPRESSION. Henry A. Lester, Division of Biology 156-29, Caltech, Pasadena, CA 91125

Functional analysis of cloned channels is being studied using two expression systems, the Xenopus oocyte and mammalian cells infected with vaccinia virus harboring the cloned channel. The two systems are complementary: the oocyte is convenient for functional analysis of site-directed mutants; the vaccinia system serves well for reconstitution of complex pathways and for high-level production of proteins for structural study. With the oocyte system, we have explored the nature of the ion conduction pathway at the nicotinic acetylcholine receptor. Channel blocking drugs, which are derivatives of local anesthetics, serve as convenient probes in conjunction with patch-clamp analysis of the mutated and expressed receptors. The data indicate that a local anesthetic derivative interacts with adjacent turns of alpha-helices within the conducting pore. This mechanism may be generally applicable to the interaction between local anesthetics and many ion channels. In our studies using the vaccinia system, both Drosophila and mammalian voltage-dependent K channels have been expressed in mammalian cells that lack endogenous K channels. The Drosophila channels retain their detailed kinetic characteristics in several different recipient cell lines and primary cells, suggesting that the primary amino-acid sequence alone is sufficient for full function.

Receptor-Modulated Transport Systems

Voltage-Gated Ion Channels

N 013 STRUCTURE AND FUNCTION OF MUSCLE CALCIUM CHANNELS. Kurt G. Beam, Department of Physiology, Colorado State University, Fort Collins, CO 80523.

We have been examining the expression of calcium channels in cultured, skeletal muscle cells (myotubes) from normal mice and from mice with muscular dysgenesis (mdg), a recessive genetic defect that eliminates excitation-contraction (E-C) coupling in skeletal muscle. Normal myotubes possess two distinct calcium currents, a fast-activating, transient current (I_{fast}) that is not blocked by dihydropyridine (DHP) derivatives and a slowly-activated, maintained current (I_{slow}) that is blocked by DHP's. Because removal of extracellular calcium does not abolish E-C coupling it seems that neither I_{fast} nor I_{slow} plays a direct role in E-C coupling. Rios and Brum have proposed[1] that the skeletal muscle DHP receptor (the slow calcium channel or a closely related protein) represents the "voltage-sensor" for E-C coupling. Consistent with this idea, we found[2] that the mdg mutation specifically eliminates I_{slow} in skeletal muscle cells without affecting calcium currents in other tissues. Working in collaboration with Drs. J. Powell, T. Tanabe and S. Numa, we carried out a molecular genetic analysis of mdg/mdg mice. This analysis revealed[3] that muscular dysgenesis is associated with alterations of the gene encoding the mouse skeletal muscle DHP receptor, and that as a result mRNA levels for this protein are strongly reduced in mdg/mdg skeletal muscle. To determine if the muscular dysgenic defects could be corrected by supplying normal copies of the skeletal muscle DHP receptor gene, we injected nuclei of cultured dysgenic myotubes with an expression plasmid carrying the entire protein coding sequence for the rabbit skeletal muscle DHP receptor. We found[3] that such injections were able to restore both E-C coupling and the I_{slow} calcium current. This result strongly supports the hypothesis that the skeletal muscle DHP receptor functions both as the slow calcium channel and as an essential component of E-C coupling.

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N 014 SPECIFICITY OF RECEPTOR/EFFECTOR COUPLING. David E. Clapham, Mayo Foundation, Rochester, MN 55905.

Over 80 receptors are coupled to ion channels or enzymes via guanine nucleotide binding proteins. How the specificity of agonist/receptor interaction is maintained in the intracellular response is a major area of interest. In cardiac atrial cells, at least three receptor types are linked via a pertussis toxin-sensitive G protein to the same ion channel. Furthermore, there is evidence for channel activation by both G protein α and $\beta\gamma$ subunits. $G_{\beta\gamma}$ subunits may release intermediate messengers such as arachidonic acid. Other cardiac channels may be activated even more indirectly by fatty acids and phospholipids. The specificity of receptor/effector coupling will be discussed with the cardiac inward rectifying K^+ channel and the muscarinic-activated phosphatidylinositol pathway in transfected cell lines as models for the study of signal transduction.

N 015 STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE SHAKER K^+ CHANNEL. William J. Kimmerly, Diane M. Papazian, Leslie C. Timpe, Ehud Isacoff, Yuh Nung Jan, and Lily Yeh Jan. Howard Hughes Medical Institute & Dept. of Physiology, University of California, San Francisco, CA 94143.

The Shaker gene of the fruit fly Drosophila melanogaster encodes a voltage-gated, fast inactivating potassium ion channel called the A channel. Like other voltage-gated cation channels, the Shaker channels contain intrinsic voltage sensors which detect electrical potential across the cell membrane. Channel gating is thought to occur when the intrinsic voltage sensor is displaced by depolarization of the membrane leading to conformational changes which open the channel. Subsequent to channel opening, the Shaker channels enter an inactivated conformation and remain inactivated as long as membrane depolarization persists. The intrinsic voltage sensors are thought to correspond to charged or polarizable amino acids located within the membrane field. It has been proposed that the basic amino acids of the putative transmembrane domain S4 may function as voltage sensors. We have changed each of the basic residues of the S4 domain to another basic residue, or to the neutral amino acid glutamine. Some of these mutants show altered voltage sensitivity of macroscopic current activation and inactivation. In contrast, these mutations have no obvious effect on other functional properties such as ion selectivity or recovery from inactivation. These results support the notion that the S4 structure plays an important role in voltage gating.

The Shaker gene encodes several alternatively-spliced mRNA species. The different mRNA species share a common core region which encodes six putative membrane-spanning domains. Conversely, each member of the class of Shaker mRNAs contains different exons which encode variant hydrophilic N- and C-terminal domains that flank the common hydrophobic core. This assortment of exons exhibited by Shaker transcripts may provide some functional diversity of K^+ channels. The involvement of these hydrophilic terminal domains in channel inactivation and other properties is being tested by different mutagenesis schemes. Several groups have isolated Sh-related genes in Drosophila, rat, mouse, and other organisms by low-stringency hybridization. Genes thus identified show 40-74 % amino acid identity with the Shaker channel. Therefore, another mechanism of functional diversification of K^+ channels relies on multiple genes. Comparison of the Shaker core amino acid sequence to other Shaker-related channel genes has identified a highly variable region which is modeled to form an extracellular loop connecting the first two putative transmembrane domains. We are analyzing the fuctional role of this domain by constructing a series of Shaker derivatives which differ only in this extracellular loop, followed by electrophysiological analysis of channels formed by injection of mRNA into Xenopus oocytes. Preliminary results suggest that this hydrophilic domain may affect the voltage dependence of channel activation.

Regulation of Intracellular Calcium

N 016 Ca^{2+} IMAGING IN SINGLE CELLS: NOVEL INSIGHTS INTO THE ROLE OF Ca^{2+} AND SIGNAL TRANSDUCTIONS, Fredric S. Fay, Ph.D., Department of Physiology, Biomedical Imaging Group, University of Massachusetts Medical Center, Worcester, MA 01655. Ca^{2+} is a ubiquitous second messenger involved in the control of many cell processes. In order to understand its role in the regulation of cell function, we have analyzed the spatial and temporal patterns of Ca^{2+} signals generated in single smooth muscle cells and eosinophils in response to contractile and chemostatic stimuli respectively. In response to contractile stimuli, $[Ca^{+2}]$ within the cytoplasm of smooth muscle cells rises prior to the onset of contraction. The magnitude of the Ca^{2+} signal is subject to powerful feedback mechanisms that limit the amplitude of the Ca^{2+} signal. This feedback is mediated at least in part by calmodulin dependent protein kinases that enhance Ca^{2+} reuptake and inhibit Ca^{2+} entry into the cytoplasm. High speed imaging of $[Ca^{+2}]$ during activation reveals that there is a local $[Ca^{+2}]$ gradient subjacent to the plasma membrane where it may be 2 - 3 times higher than in the cytoplasm. This may result from asymmetries in the distribution of Ca^{2+} storage and reuptake systems as 3D molecular mapping of Ca^{2+} regulatory molecules reveals that the high capacity low affinity Ca^{2+} storage protein calsequestrin is localized almost exclusively beneath the surface membrane whereas sarcoplasmic reticulum Ca ATPases are more uniformly distributed throughout the smooth muscle cell. In eosinophils during migration towards a chemostatic stimulus, there is also an obligatory increase in $[Ca^{+2}]$. Here too the $[Ca^{+2}]$ signal is spatially heterogeneous. The pattern of $[Ca^{+2}]$ changes is related to local changes in cell behavior that underly the chemotaetic response. $[Ca^{+2}]$ at the motile leading edge of the cell is slightly higher than that directly behind it. The highest $[Ca^{+2}]$ is found in the midregion of the cell. Changes in cell direction are preceded by a transient rise in $[Ca^{+2}]$ in the new direction of motion. The relationship of the $[Ca^{+2}]$ gradients to known cycles of polymerization and depolymerization of cytoskeletal proteins during chemotaxis will be discussed. Supported in part by grants from the NIH (HL14523) and the NSF (5-22528).

N 017 IP_3 ACTIVATED CALCIUM CHANNEL FROM BRAIN, Christopher D. Ferris and Solomon H. Snyder, Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205. Inositol 1,4,5-trisphosphate (IP_3), a major second messenger molecule for the actions of hormones, neurotransmitters and growth factors, releases calcium from intracellular non-mitochondrial stores. Binding sites for [^{3}H]IP_3 have been pharmacologically characterized in brain membranes(1) and have been mapped in the brain by autoradiography(2). An IP_3 receptor protein has been purified from rat cerebellum(3) and localized by immunohistochemical techniques to specific sites in the endoplasmic recticulum (ER), including portions of the ER which surrond the cell nucleus(4). Western blot analysis of a variety of tissues indicates that the IP_3 receptor protein is essentially ubiqitous, though it is most prevalent in the brain. IP_3 stimulated Ca^{2+} release is selectively regulated by stoiciometric phosphorylation of the IP_3 receptor by cAMP dependent protein kinase(5). The purified IP_3 receptor has been recently reconstituted into lipid vesicles and shown to selectively mediate IP_3 stimulated $^{45}Ca^{2+}$ flux(6). Thus, the purified IP_3 receptor contains both the IP_3 recognition site as well as the associated calcium channel(6). IP_3 receptor mediated calcium flux in reconstituted lipid vesicles is allosterically regulated by adenine nucleotides, and this regulation is conferred through an adenine nucleotide binding site on the purified IP_3 receptor(7). Thus, the IP_3 receptor resembles the ryanodine receptor of skeletal muscle in its function as a calcium release channel in ER-like structures, its proposed tertiary structure, parts of its predicted amino acid sequence, and in its having an adenine nucleotide regulatory site.

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N 018 THE RYANODINE-SENSITIVE CA^{2+} RELEASE CHANNEL OF SKELETAL MUSCLE IS A CA^{2+}-GATED, COOPERATIVELY COUPLED HOMOTETRAMER.
Gerhard Meissner, F. Anthony Lai, Le Xu and Hee-Bong Lee.
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Skeletal muscle contraction is initiated by the rapid release of Ca^{2+} ions through Ca^{2+} release channels localized in an intracellular membrane compartment, the sarcoplasmic reticulum (SR). The Ca^{2+} release channel has been identified as the receptor for the plant alkaloid ryanodine, and purified to apparent homogeneity as a 30S protein complex comprised of polypeptides of M_r 565,223 (Lai *et al.* 1988 *Nature* 331, 315; Takeshima *et al.* 1989 *Nature* 339, 439). Although the mechanism of *in vivo* Ca^{2+} release has not yet been fully defined, rapid mixing vesicle-ion flux and planar lipid bilayer single-channel measurements have indicated that the SR Ca^{2+} release channel is regulated by Ca^{2+}, adenine nucleotides, Mg^{2+} and calmodulin, as well as by exogenous ligands including the two Ca^{2+} releasing drugs ryanodine and caffeine (Rousseau *et al.* 1988 *Arch. Biochem. Biophys.* 267, 75).

Recently, we have further characterized the SR Ca^{2+} release channel by determining the subunit structure of the purified complex, and high- and low-affinity [^{3}H]ryanodine binding to the membrane-bound receptor in the absence and presence of Ca^{2+} (Lai *et al.* 1989 *J. Biol. Chem.* 264, 16776), as well as by reconstituting the purified 30S complex into planar lipid bilayers and lipid vesicles. These studies have suggested that the ryanodine receptor-Ca^{2+} release channel complex of skeletal muscle sarcoplasmic reticulum is a negatively charged, cooperatively coupled and Ca^{2+}-gated homotetramer.

Supported by NIH and Muscular Dystrophy Association.

Receptor-Modulated Transport Systems

Hormonal Control of Glucose Transport

N 019 REGULATION OF GLUCOSE TRANSPORTER GENES IN VIVO AND IN VITRO, Mike Mueckler, Karen Tordjman, Konrad Keller, Laszlo Koranyi, Raymond E. Bourey, and M. Alan Permutt, Departments of Cell Biology and Physiology, and Medicine, Washington University School of Medicine, St. Louis, MO 63110.
Insulin-sensitive tissues express two species of facilitative glucose transporter. The human GLUT 1 and rat GLUT 4 transporters were cloned from HepG2 cells and adipose/heart tissues, respectively. Based largely on the amino acid sequences deduced from these clones, both transporters are predicted to possess very similar structures in which the polypeptides fold in and out of the membrane twelve times. Several of the putative transmembrane segments may form moderately amphipathic helices that comprise the walls of a hydrated channel through which glucose transverses the membrane. Expression of the GLUT 1 and GLUT 4 mRNAs in Xenopus laevis oocytes confirmed the functional identity of the clones. Kinetic analysis of transport in oocytes indicates that the two transporters exhibit distinct kinetic properties consistent with their behavior in the native cell types.

The tissue distribution of the two transporters and regulatory studies suggest that the GLUT 1 product plays a housekeeping role in the provision of glucose for the needs of the individual cell, whereas the GLUT 4 protein likely plays a more philanthropic function in decreasing blood glucose for the benefit of the organism. Thus, expression of the GLUT 4 transporter appears to be restricted to insulin-sensitive tissues, whereas the GLUT 1 transporter is widely expressed in mammalian tissues. Glucose deprivation of murine 3T3-L1 adipocytes increases expression of the GLUT 1 mRNA and protein but not the GLUT 4 gene products. Expression of the GLUT 1 transporter, but not the GLUT 4 protein, is also directly induced by chronic insulin and sulfonylurea treatment of these cells. Acutely, both transporters are capable of insulin-induced translocation from an intracellular compartment to the plasma membrane, although a much greater proportion of GLUT 1 transporter is found in the plasma membrane in the absence of insulin. In contrast, in vivo studies indicate that the level of GLUT 1 protein in rat soleus muscle is largely unaffected by various treatments that result in perturbations in blood glucose and insulin levels, whereas expression of GLUT 4 protein in this tissue appears to correlate inversely with blood glucose levels. Expression of the GLUT 1 transporter in rat brain is also inversely correlated with blood glucose levels. Taken as a whole these studies are consistent with the proposed physiologic roles of the GLUT 1 and GLUT 4 glucose transporters.

N 020 INTRACELLULAR TRANSPORT OF VESICLES CONTAINING THE INSULIN RESPONSIVE GLUCOSE TRANSPORTER (MUSCLE/FAT ISOTYPE, GLUT 4) Paul F. Pilch, Natalio Kotliar, Robert Del Vecchio, Wells Wilkinson and Galini Thoidis, Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118.

Facilitative glucose transporters comprise a multi-gene family of five structurally related proteins one of which is restricted in its expression to tissues, namely muscle and fat, that respond acutely and dramatically to insulin in elevating their rate of glucose uptake. Previous studies have shown that the increase in glucose uptake due to insulin is a result of transporter movement from an intracellular site to the cell surface, a process called transporter translocation. We have recently shown that the insulin-dependent increase in glucose transport seen in rat fat cells is a result of the translocation of the two transporter isoforms expressed in these cells, the erythroid and muscle/fat isotypes. However, the behavior of the two isoforms differ in that only the latter species is restricted to an intracellular site in resting cells, and its insulin-dependent movement accounts for almost all of the increase in glucose uptake seen after cellular exposure to insulin. This scenario also applies to human fat cells as we just experimentally verified. Thus, it appears that the muscle/fat glucose transporter isoform is specifically targeted to a unique intracellular vesicle whose movement is absolutely insulin dependent. Our current experimental efforts are directed towards describing the biochemical properties of the translocating vesicles and how these may be modified in response to insulin. Immunoadsorption of intracellular vesicles with an antibody directed against muscle/fat transporter isoform following a variety of physiological and biochemical manipulations has revealed the following: 1. The vesicles contain a relatively small number of proteins as determined by silver staining. 2. A number of vesicle proteins are phosphoproteins including the glucose transporter. We have not observed dramatic changes in these phosphoproteins following cellular insulin exposure. 3. The vesicles contain GTP binding proteins. In order to determine if these vesicles are needed to express the dramatic increases in glucose transport or if expression of the GLUT 4 protein alone would do the trick, we transfected GLUT 4 cDNA into the well differentiated C2/C12 skeletal muscle cell line. Expression of the cDNA was verified by Northern blot analysis and expression of protein was verified by immunoblotting. However, the transfected cells showed no insulin-dependent increase in glucose uptake. Our results, taken together with the published results of other research groups, suggest that manifestation of the glucose transporter insulin response characteristic of fat cells (and most probably muscle) will require the expression of a group of genes whose expression is required for the formation of the insulin-sensitive, GLUT 4-containing vesicles.

Receptor-Modulated Transport Systems

N 021 Hormonal Regulation of Glucose Transport in Adipose Cells.
I.A. Simpson, S.W. Cushman, J.J. Egan, A.H. Habberfield, C. Londos, H. Nishimura, J. Saltis. NIDDK, NIH, Bethesda, Md. 20892.

Glucose transport is regulated by three distinct hormonal systems which interact in the isolated rat adipose cell. The primary regulator of glucose transport activity is insulin which induces the translocation of two distinct glucose transporters, GLUT1 and GLUT4, from an intracellular pool to the plasma membrane. A conceptually similar mechanism exists for the stimulation of glucose transport activity by hormones/agents such as vasopressin, oxytocin and (PMA). The enhanced glucose transport activity elicited by these latter agents is attributed to the activation of protein kinase C, either directly or by the initial activation of a phospholipase C, which in turn again mediates the translocation of both glucose transporter species. The time course for the stimulation of glucose transport activity is slower and the magnitude of the response is only ≈ 10% of that seen with insulin suggesting that different mechanisms might be responsible for the respective translocations. However, we have recently demonstrated that insulin itself significantly stimulates protein kinase C and thus raising the possibility that the activation of C-kinase might play an intermediate role directly in the mechanism of insulin action.

The third class of hormones that modulate glucose transport activity comprises the lipolytic and antilipolytic hormones. Lipolytic hormones such as ß-adrenergic agonists, ACTH and glucagon inhibit maximally insulin-stimulated glucose transport activity by ≈ 50% as well as decreasing the sensitivity of the cells to insulin. Conversely, antilypolytic agents such as adenosine, PGE_1, and nicotinic acid augment insulin-stimulated glucose transport activity by ≈ 40%, completely block the actions of the lipolytic agents, and enhance the sensitivity of the cells to insulin. Neither group of hormones appears to act through a cAMP-mediated mechanism or through alterations in the phosphorylation state of GLUT1 or GLUT4. However, the involvement of the guanine nucleotide binding proteins G_s and G_i is implied by the loss of corresponding hormone responsiveness following treatment with cholera and pertussis toxins. The actions of vasopressin are also significantly altered by these same toxins. Thus, mechanisms are now apparent to coordinate the various functions of the adipose cell to produce an integrated glucose transport response.

Pathways and Mechanisms of Protein Sorting

N 022 INTERACTION OF MITOCHONDRIAL IMPORT SIGNALS WITH NATIVE AND ARTIFICIAL MEMBRANES, Michael G. Douglas✦, Douglas M. Cyr✦, and David W. Hoyt✣, ✦Department of Biochemistry and Biophysics, UNC Medical School at Chapel Hill 27514 and ✣Department of Biochemistry, UT Southwestern Medical Center, Dallas, Texas 75235

Import competent signals for mitochondrial import are capable of forming positively charged amphipathic α-helical structures. Little information, however, is available regarding their initial interaction with the membrane translocation apparatus. We have chemically synthesized, using either all l- or all d- aminoacids, a 19 residue peptide which constitutes the presequence of the F1β-subunit precursor. We have characterized the ability of these highly purified d- and l- peptides to bind to both mitochondrial and artificial membranes of similar composition. These studies reveal that both all d- and all l- presequence peptides bind to phospholipid vesicles and adopt a predominantly α-helical structure. The d- peptide exhibits a greater tendency to adopt an α-helical structure than the l- peptide. This suggests that the peptides maybe sensitive to the chirality of the phospholipid. The F1β presequence peptide associates strongly ($\Delta\Pi$max 8.2 mN/meter) with the phospholipid monolayers causing an increase in the surface pressure. Although these data are consistent with insertion, they do not rule out the possibility that the change in surface pressure is caused by peptide binding at the surface. Consistent with this, we observe that the F1β presequence peptide is not able to cause release of carboxy fluoroscene from loaded phospholipid vesicles or influence the membrane potential in transport competent mitochondria. Finally, both peptides failed to inhibit binding and import of several precursors at peptide concentrations less than 20μM. These data indicate that the competition by presequence peptide within the translocation apparatus for protein transport may occur not at the membrane surface but below the surface.

N 023 DEFINING COMPONENTS, IN VITRO AND IN VIVO, REQUIRED FOR TRANSPORT FROM THE ER. S. Ferro-Novick, M.E. Groesch, R. Bacon, A. Newman, H. Ruohola, J. Shim, G. Rossi, M. Clague and J. Graf, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510.

An understanding of transport from the ER through the Golgi complex will require the isolation of functional intermediates as well as a molecular identification of components required at this stage of the pathway. We have isolated a fusion competent vesicular intermediate in ER to Golgi transport in yeast. Analysis of the formation and utilization of this intermediate has led to the following model. Donor permeabilized yeast cells, lacking functional Golgi membranes, release carrier vesicles containing the 26kD core glycosylated form of pro-α-factor. These vesicles bind to and fuse with the Golgi apparatus to transport pro-α–factor to a compartment in the Golgi complex where additional glycosylation occurs to produce a high molecular weight species. Our results suggest that the formation and utilization of vesicles are two distinct biochemical events in vitro. Studies have shown that the 26kD form of pro-α–factor, contained within vesicles, is converted to a 28kD species. Conversion of the 28kD form of pro-α–factor to the final reaction product requires the GTP-binding protein, Ypt1(Bacon et al., JCB 109: 1015, 1989). Ypt1 may function in conjunction with BET and SEC gene products. We have shown that YPT1 genetically interacts with BET2 (Newman and Ferro-Novick, JCB 105:1587; 1987) and less strongly with SEC21, two gene products implicated in ER to Golgi transport. We have also established a complex pattern of genetic interactions among BET1, SEC22, and BOS1, a newly identified gene required for ER to Golgi transport. SEC21 is a fourth member of this group of interacting genes and is less strongly involved in these interactions. Now that we have raised antibodies to several of these gene products, we are performing biochemical studies to determine the nature of these genetic interactions and their relationship to the steps we have defined in vitro.

N 024 GTP FUNCTION DURING PROTEIN TRANSLOCATION ACROSS THE ENDOPLASMIC RETICULUM. Reid Gilmore, Peter Rapiejko and Tim Connolly . Department of Biochemistry, University of Massachusetts Medical School, Worcester, MA 01655. The signal recognition particle (SRP) mediated transport of proteins across mammalian endoplasmic reticulum requires GTP in a capacity distinct from polypeptide elongation. We examined the role of GTP in this process by a molecular characterization of translocation intermediates that accumulate after incubation of SRP-ribosome-nascent chain complexes with microsomal membranes. SRP-receptor catalyzed displacement of SRP from the signal sequence of the nascent chain was GTP-dependent both with intact membranes and with the purified SRP receptor. The SRP receptor contains amino acid sequences which are similar to guanine ribonucleotide binding site consensus sequence elements. Interestingly, homologous amino acid sequence elements have also been identified in the 54 kD subunit of SRP by the laboratories of Peter Walter and Bernard Dobberstein. The GTP hydrolysis cycle of the SRP receptor was further analyzed by characterizing a SRP-SRP receptor complex that is formed in the presence of nonhydrolyzable GTP analogues. Nonhydrolyzable GTP analogues will inhibit protein translocation by sequestering the SRP in a high ionic strength-insensitive complex with the SRP receptor. Purified preparations of the SRP receptor, but not SRP, hydrolyze GTP at a low but detectable rate. GTP hydrolysis by the SRP receptor was stimulated approximately 10-fold in the presence of equimolar concentrations of SRP. Current evidence indicates that only one a single guanine ribonucleotide is bound per SRP-SRP receptor complex during a GTP hydrolysis cycle. Evidence will be presented that indicates that the affinity of SRP for the SRP receptor is regulated by the presence of bound guanine ribonucleotides. These alterations in binding affinity are proposed to control the specificity and directionality of ribosome targeting to the endoplasmic reticulum.

Receptor-Modulated Transport Systems

Mechanisms and Regulation of Intracellular Protein Transport

N 025 COATED VESICLE ADAPTORS. Margaret S. Robinson, University of Cambridge, Department of Clinical Biochemistry, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, England.

Adaptors are the protein complexes that link clathrin to the cytoplasmic domains of transmembrane proteins (e.g. receptors) in coated pits and vesicles. Two major types of adaptors have been identified: one for the plasma membrance and one for the Golgi apparatus. Both consist of heterodimers of ~100-kd proteins called adaptins, plus two associated smaller proteins. The plasma membrane adaptor contains an α-adaptin and a ß-adaptin, while the Golgi adaptor contains a γ-adaptin and a ß-adaptin. Both α and ß-adaptins have recently been cloned and show no apparent sequence homology, although both appear to have a similar two-domain structure: a ~70-kd N-terminal domain connected by a proline and glycine-rich hinge to a ~30-kd C-terminal domain. I have now cloned cDNAs encoding γ-adaptin by screening a λgt11 bovine brain expression library with a mixture of two monoclonal anti-γ antibodies, generously provided by E. Ungewickell (Ahle et al., 1988, EMBO J. 7: 919-929). Positive clones were then used to obtain the full-length sequence from a mouse brain cDNA library. The N terminus of γ-adaptin and several of its CNBr fragments were sequenced and used to confirm the identity of the clones. The deduced protein sequence of γ-adaptin was found to be homologous to that of α-adaptin, with several stretches of identical amino acids. Like α and ß-adaptins, γ-adaptin has a proline and glycine-rich hinge region, dividing it into N and C-terminal domains. Thus, it should now be possible to use genetic engineering to exchange domains between α and γ-adaptins, in order to try to find out how adaptors are targeted to the appropriate membrane compartment of the cell, and how they recruit the appropriate receptors into the coated vesicle.

N 026 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.

Membrane Transport in Disease

N 027 CHLORIDE TRANSPORT IN CYSTIC FIBROSIS, W.H. Cliff, R.T. Worrell & R.A. Frizzell, Dept. Physiol. & Biophys., Univ. Alabama at Birmingham, Birmingham, AL 35294.

Abnormal regulation of epithelial Cl conductance is a diagnostic feature of the genetic disease cystic fibrosis (CF). Single-channel patch-clamp studies have identified an outwardly-rectified, 40-50 pS Cl channel that is activated by protein kinases A and C in normal but not CF epithelia, implicating it as the locus for defective regulation by second messenger pathways. This channel is blocked by stilbene disulfonates such as DIDS (1). We examined the properties of Cl conductances in normal and CF secretory epithelial cells that are activated by different classes of agonists using the whole-cell patch-clamp technique. Agonists that activate cAMP-mediated Cl secretion elicit a whole-cell Cl current (I_{Cl}^{cAMP}) that is linear over the range ±100 mV and shows no time dependence during a voltage pulse. I_{Cl}^{cAMP} is not affected by addition of the DIDS to the bath (100 μM). Ca ionophores elicit a Cl current (I_{Cl}^{Ca}) whose current-voltage relation is outwardly-rectified and shows time-dependent activation at depolarizing voltages (>+40 mV) and time-dependent inactivation at hyperpolarizing voltages (<-40 mV). The outward Cl currents activated by Ca are inhibited by 50 μM DIDS. Cell swelling activates a Cl current (I_{Cl}^{swell}) that is also outwardly-rectified, but differs from I_{Cl}^{Ca} in its voltage-dependent kinetics (i.e., activation at hyperpolarizing voltages, inactivation at depolarizing voltages). Outward Cl currents evoked by swelling are also DIDS-sensitive (K_i ~ 5 μM). These results indicate that cAMP, Ca and cell swelling activate different Cl conductances which can be distinguished on the basis of their current-voltage relations, their time- and voltage-dependent kinetics and their blocker sensitivities. The single Cl channels responsible for I_{Cl}^{swell} have been identified by biophysical and kinetic criteria (2). The finding that Cl currents activated by cAMP and Ca have different conductance and kinetic properties and blocker sensitivities argues for regulation of different Cl channels. The properties of I_{Cl}^{Ca} most closely resembles the outwardly-rectified Cl channel associated with Cl secretion. I_{Cl}^{cAMP} is ohmic, voltage-independent and DIDS-insensitive. These properties are difficult to reconcile with those of the outward-rectifier and suggest that the single-channel basis for cAMP-stimulated Cl conductance is not yet completely defined.

1. Bridges, R.J., R.T. Worrell, R.A. Frizzell and D.J. Benos (1989). Am. J. Physiol. 256(Cell Physiol. 25):C902-C912.
2. Worrell, R.T., A.G. Butt, W.H. Cliff and R.A. Frizzell (1989). Am. J. Physiol. 256(Cell Physiol. 25):C1111-C1119.

N 028 MOLECULAR GENETICS OF CYSTIC FIBROSIS, John R. Riordan, The Hospital for Sick Children and University of Toronto, Toronto, Ont., Canada M5G 1X8.

Cystic Fibrosis (CF) is the most common fatal genetic disease in the Caucasian population (approximately 1/2500 live births). It is an autosomal recessive condition in which heterozygotes (about 5% of this population) are disease free. The regulation of transepithelial salt and water movement across the walls of the ducts of exocrine tissues such as the pancreas and sweat glands and the airways of the lung is abnormal. As a result, these passages become obstructed by inadequately hydrated macromolecular mucous-containing secretions. This has the most severe consequences for the pancreas which becomes unable to secrete digestive enzymes and the lungs which become congested and colonized by opportunistic bacteria, primarily Pseudomonas aeruginosa. Death normally occurs due to the latter condition. Currently, lung transplantation is being performed in a limited number of cases. In 1989 the gene which is mutated in CF was identified and cloned on the basis of its chromosomal localization and tissue specific expression in epithelia known to be affected in the disease. Transcription of the 250 Kb gene produces a 6.5 Kb mRNA in cells of such tissues. The sequence of corresponding cDNAs predicted a protein product with distinctive structural features. Hence, the predicted 1480 residues should constitute 3 different domain types: a membrane associated portion consisting of 6 transmembrane segments and a cytoplasmic nucleotide binding domain followed by a unique highly charged 'R'-domain which links the first two domains to two more which are similar. On the basis of these predictions, the CF gene belongs to a super family of membrane transporter genes which contains P-glycoprotein multidrug transporters, the yeast 'a' mating factor transporter (STE-6), several prokaryotic peptide and glycan transporters, many bacterial nutrient transporters and others. The CF gene is apparently itself an ancient member of this super family as a highly conserved analogue is expressed in elasmobranchs. Present studies are directed at the identification of the precise role of the CF gene product (CFTR) in epithelial ion transport and the influence of mutations at different sites in the sequence on this function.

N 029 GLUCOSE TRANSPORT: FROM MOLECULES TO MAN, Ernest M. Wright, Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90024-1751.

In the kidney and intestine, sugar transport involves both the Na^+-dependent and facilitated glucose transporters. The Na^+/glucose cotransporter is responsible for the uphill transport of glucose from lumen to cell across the brush border membrane, and the facilitated transporter is responsible for the downhill transport from cell to blood across the basolateral membrane. Both transport proteins have been cloned, sequenced, and expressed in model systems. There is no structural, immunological, or functional homology between the facilitated and Na-cotransport proteins.

Genetic defects in glucose transport have been identified, such as renal glycosuria and intestinal glucose-galactose malabsorption syndrome. These appear to involve the renal and intestinal brush border Na^+/glucose cotransporter. However, the defects are unrelated, and it has been concluded that two different genes are involved. We are currently attempting to clone the renal Na^+/glucose cotransporter, to map the gene, and to identify the molecular defects in both the renal and intestinal Na^+/glucose cotransporters. Preliminary evidence suggests that a single point mutation can account for glucose-galactose malabsorption.

Receptor-Modulated Transport Systems

Poster Session-I

N 100 THE SODIUM/IODIDE SYMPORTER: EXPRESSION IN OOCYTES AND STUDIES WITH INHIBITORS. Nancy Carrasco, Franck Vilijn, and Stephen Kaminsky. Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, 10461.

The iodide carrier of the thyroid gland is a membrane protein responsible for the active, Na^+-dependent accumulation of iodide by the thyroid follicular cells, a process induced by TSH via cAMP. Iodide uptake is the first step in the synthesis of thyroid hormones T3 and T4. We report the expression of iodide transport activity in *Xenopus laevis* oocytes microinjected with mRNA isolated from FRTL-5 cells, a line of highly differentiated rat thyroid cells whose growth and iodide uptake activity are markedly dependent on TSH. A 7-fold increase of Na^+-dependent, ClO_4^- sensitive iodide transport activity (an average of 3.5 pmoles of [125]iodide per oocyte in 45 min) is observed in oocytes microinjected with mRNA from FRTL-5 cells maintained in the presence of TSH over control oocytes (~0.5 pmoles), including those microinjected with mRNA from FRTL-5 cells maintained in the absence of TSH. Sucrose gradient fractionation of mRNA followed by injection of fractions revealed that the mRNA encoding the transporter is 2.4 to 4.0 kb in length.

Iodide transport in plasma membrane vesicles is shown to be inhibited by harmaline-like compounds and kinetic studies indicate they compete with sodium.

N 101 CLONING AND CHARACTERIZATION OF A cDNA ENCODING A POSSIBLE POTASSIUM CHANNEL ISOLATED FROM HUMAN BRAIN, Simon N. Freeman[1], John C. Brennand[2], Steven Hall[2], Victoria Wilson[1], Karen A. Mileham[1], Helen George[1], Allan W. Winter[1], Edward C. Conley[1], Alison Davies[2], Neil French[2], Norman J. W. Russell[2] and William J. Brammar[1], [1] ICI/Joint Laboratory, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, U.K. and [2] Biotechnology 1, ICI Pharmaceuticals PLC, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K.

PCR technology was used to amplify the entire coding region of MBK-1, a mouse brain voltage-dependent potassium channel cDNA (Tempel, et al., 1988), from mouse brain total RNA. The PCR-amplified cDNA was cloned and used to screen a human brain cDNA library under conditions of reduced stringency. Using this method we isolated two identical cDNAs, HKC-1 and HKC-19, which exhibited 93% sequence homology to the S4-like voltage sensor transmembrane domain of MBK-1. The presence of a S4-like sequence in the human cDNAs suggests that HKC-1 and HKC-19 encode for a voltage-dependent potassium channel. The human cDNA is being used to screen a human brain cDNA library under conditions of high stringency to isolate full length clones which will then be used fro expression studies in Xenopus oocytes and to characterize the expression of the corresponding genes in human tissues by in situ hybridization.

N 102 PRODUCTION OF RECOMBINANT HUMAN MULTIDRUG TRANSPORTER IN A BACULOVIRUS EXPRESSION SYSTEM, Ursula A. Germann, Mark C. Willingham, Stephen J. Currier, Shigeo Tanaka, Ira Pastan and Michael M. Gottesman, NCI, NIH, Bethesda, MD 20892

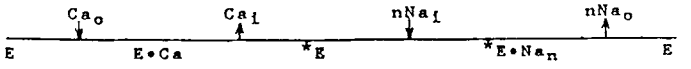
The plasma membrane-associated human multidrug resistance (*MDR*1) gene product, known as the 170-kDa P-glycoprotein or the multidrug transporter, acts as an ATP-dependent efflux pump for various cytotoxic agents. The mechanisms of drug-binding and the transport process are, however, poorly understood. To further investigate its structure and function, we overproduced the human multidrug transporter in insect cells after infection with a recombinant baculovirus. Using immunocytochemical methods, high amounts of recombinant multidrug transporter were detected 2-3 days after infection on the external surface of the plasma membranes, in the Golgi apparatus, and within the nuclear envelope. The recombinant protein expressed in insect cells is not susceptible to endoglycosidase F treatment and has a lower apparent M_r of 140,000 corresponding to the non-glycosylated precursor of its authentic counterpart synthesized in multidrug-resistant cells. Labeling experiments indicated that the recombinant multidrug transporter is post-translationally modified by phosphorylation and can be photoaffinity-labeled by [^{3}H]azidopine. Various drugs and multidrug-resistance agents (e.g., daunomycin > verapamil > vinblastine ≈ vincristine) compete with the [^{3}H]azidopine binding reaction when added in excess, indicating that the recombinant human multidrug transporter expressed in insect cells is functionally similar to its authentic counterpart.

N 103 BIOENERGETICS AND FUNCTIONAL RECONSTITUTION OF THE GABA-CARRIER OF SYNAPTIC VESICLES, Johannes W. Hell., Peter R. Maycox and R. Jahn, Department of Neurochemistry, Max-Planck-Institute for Psychiatry, Am Klopferspitz 18a, D-8033 Martinsried,FRG

Energy dependent GABA uptake by rat brain synaptic vesicles is driven by an electrochemical proton gradient generated by an ATP-dependent proton pump. We have investigated which component of the electrochemical gradient is responsible for GABA uptake, the membrane potential ($\Delta\psi$) or the pH gradient (ΔpH). For this purpose, uptake was analyzed in intact synaptic vesicles and in proteoliposomes which were reconstituted by dilution after solubilisation of vesicle proteins with Na cholate. GABA uptake was observed at high and low Cl^- concentrations, i.e. under conditions whereby either ΔpH or $\Delta\psi$ is the predominant driving force. Application of NH_4^+ which selectively abolishes ΔpH, inhibited GABA-uptake at pH 7.3 but not at pH 6.5. This indicates that under the latter condition GABA uptake is driven exclusively by $\Delta\psi$, i.e. the uptake is electrogenic. Application of SCN^- which selectively abolishes $\Delta\psi$, did not affect GABA-uptake at low concentrations suggesting that ΔpH can act as driving force. In all experiments, the properties of the reconstituted system were very similar to those of intact synaptic vesicles. Together, these properties clearly distinguish GABA uptake from vesicular glutamate uptake which is solely driven by $\Delta\psi$, and from monoamine uptake which is more strongly dependent on ΔpH. We conclude that GABA uptake is electrogenic and probably occurs in exchange for protons.

N 104 CONSECUTIVE MECHANISM OF CATION TRANSPORT IN THE CARDIAC Na-Ca EXCHANGE SYSTEM, Daniel Khananshvili, Department of Biochemistry, Weizmann Institute of Science, Rehovot, P.O.B. 26, 76100, Israel.

In order to distinguish between the Sequential and Consecutive mechanisms of Ion transport in the Na-Ca exchange system the initial rates of the Na_i-dependent ^{45}Ca-uptake were measured under zero-trans conditions ($[Ca]_i = 0$, $[Na]_o = 0$) by using the reconstituted proteoliposomes with entrapped EGTA. With a fixed $[Na]_i$ = 10-160 mM and varying $[Ca]_o$ = 2.5-122 μM for each $[Na]_i$, the observed values of K_m and V_{max} increased proportionally from 7.7 μM to 33.5 μM and from 2.3 nmol$\cdot mg^{-1}\cdot sec^{-1}$ to 9.0 nmol$\cdot mg^{-1}\cdot sec^{-1}$ respectively. The V_{max}/K_m values show only + 2-10% deviation from the average value of 0.274 nmol$\cdot mg^{-1}\cdot sec^{-1}\cdot\mu M^{-1}$ over the whole range of $[Na]_i$. These deviations are within the standard error for the assay of V_{max} (+ 3-7%), K_m (+ 11-17%) and V_{max}/K_m (+ 11-19%). Thus under conditions in which the V_{max} and K_m values undergo 4-5 fold changes, the V_{max}/K_m ratios are constant. Under conditions with a very low fixed $[^{45}Ca]_o$ = 1.1 μM ($<< K_m$) and varying $[Na]_i$ = 10-160 mM, the initial rates of the Na_i-dependent ^{45}Ca-uptake are independent of the $[Na]_i$. These data are consistent with the consecutive (Ping-Pong) mechanism of cation transport in this system:



N 105 ROLE OF CALMODULIN IN THE REGULATION OF THE PLASMA MEMBRANE Ca^{2+}-ATPase, D. Kosk-Kosicka[1], T. Bzdega[1] & R.F. Steiner[2], University of Maryland, Department of Biological Chemistry, School of Medicine, Baltimore, MD 21201[1], and Department of Chemistry, Catonsville, MD 21228[2].

The subject of our studies is the role of the regulatory protein, calmodulin in regulation of the erythrocyte Ca^{2+}-ATPase. Using purified enzyme we have previously demonstrated a novel mechanism of Ca^{2+}-ATPase activation by oligomerization, and shown that the known activation by calmodulin is operative only on the monomeric enzyme (Kosk-Kosicka & Bzdega, JBC 263, 18184, 1988; Kosk-Kosicka et al., JBC 264, 19495, 1989).

In the present study, by employing fluorescence resonance energy transfer (FRET) between separately labeled Ca^{2+}-ATPase molecules, we have found that calmodulin added before mixing donor- and acceptor-labeled enzyme populations prevents the occurrence of energy transfer. Further, calmodulin also prevents the apparent decrease in energy transfer that is normally observed upon dilution of the labeled enzyme populations with the unlabeled enzyme. The calmodulin effect is dose-dependent, and is not mimicked by any other proteins such as BSA. Additionally, similar inhibition of FRET is observed when the C-terminal calmodulin half, as opposed to the N-terminal half, is substituted for calmodulin.

Our FRET studies, supported by the Ca^{2+}-ATPase activity data, indicate that calmodulin, by binding to both monomeric and oligomeric forms of the enzyme, prevents changes in degree of oligomerization and locks them temporarily in a given state. It appears that the equilibrium between enzyme monomers and oligomers, and the availability of calmodulin to these two forms determines the activation pathway of the erythrocyte Ca^{2+}-ATPase.

N 106 EXPRESSION OF Na DEPENDENT CHOLINE TRANSPORT IN *XENOPUS* OOCYTES FOLLOWING INJECTION OF NEURONAL mRNA, S. O'Regan, S. Birman, F.M. Meunier, Département de Neurochimie, Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 91198 Gif-sur-Yvette Cedex, France.
Cholinergic nerve terminals have been shown to transport choline by a strictly Na dependent (Li does not substitute) mechanism that is sensitive to changes in the membrane potential and the Na gradient. The choline so taken up is primarily used for the synthesis of the neurotransmitter acetylcholine. Poly A+ mRNA's were prepared from the cholinergic somata of the electric lobe, a brain region of *Torpedo marmorata*. Upon injection, *Xenopus* oocytes gained a Na dependent component of choline uptake which has a high affinity for choline and a high sensitivity to hemicholinium-3 (IC_{50}'s for both compounds ≈ 0.3 µM) but a low affinity for Na (EC_{50} ≈ 200 mM), thus resembling the high affinity choline transporter in synaptosomes from the electric organ. Na dependent choline uptake by oocytes was increased after enrichment of mRNA by a sucrose gradient, compatible with the involvement of a single peptide chain. However, non-injected oocytes also accumulate choline by a different mechanism, and which serves for phospholipid synthesis.
While not sufficiently sensitive for direct use in an expression selection cloning strategy, the reconstitution of the high affinity choline transporter in the oocyte membrane is a new way of studying this protein.(Supported by the Association Française contre les Myopathies)

N 107 HORMONAL REGULATION OF THE Na,K-ATPase MULTIGENE FAMILY IN CARDIAC MYOCYTES. J. Orlowski and J.B Lingrel, Department of Molecular Genetics, Biochemistry and Microbiology, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524

The Na,K-ATPase α isoform (α1, α2 and α3) and β subunit genes exhibit a complex pattern of expression during heart development. To identify possible molecular signals that regulate the differential expression of these genes, isolated neonatal rat cardiac myocytes were cultured in chemically-defined medium and the responses of the multiple Na,K-ATPase subunit mRNAs to various hormones were tested. Triiodothyronine (T3) acted as a positive regulator of the α2, α3 and β mRNAs without influencing the α1 mRNA abundance. In contrast, dexamethasone, aldosterone and isoproterenol selectively repressed the T3-mediated induction of α3 mRNA expression without affecting the increases in α2 and β mRNA levels. Addition of norepinephrine and carbachol to the cultures had little appreciable effect on expression of the α isoform and β mRNAs. These data indicate that multiple hormones differentially regulate the expression of the Na,K-ATPase multigene family in cardiac myocytes *in vitro* and, therefore, may also be important physiological modulators *in vivo*.

N 108 EVIDENCE FOR A K+/H+ COTRANSPORT SYSTEM IN PLASMA MEMBRANE VESICLES OF AN ACIDOPHILE ALGA, Ulrich Glaser and Uri Pick, Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel. *Dunalliela acidophila* is an unicellular halotolerant green algae which grows at pH1 while maintaining a neutral internal pH and a positive-inside membrane potential. *D. acidophila* accumulates K^+ up to 10^2 -10^3 fold with respect to the external concentration. We have studied the mechanism of K^+ uptake in reconstituted plasma membrane vesicle preparation and obtained the following results:
(1) ATP catalyses a vanadate-sensive ^{86}Rb efflux from the vesicles. ATP also catalyzes H^+ uptake and generates a positive-inside membrane potential.
(2) Protonophores inhibit the ATP dependent Rb efflux, whereas a lipophilic anion, which dissipates the ATP-induced membrane potential does not produce any inhibition.
(3)An artificially induced pH gradient, acid inside also induces ^{86}Rb efflux from the reconstittued vesicles. We conclude that the K^+ transport mechanism is via a K^+/H^+ cotransport system and not a primary H^+/K^+ ATPase or a K^+ channel. Such a mechanism enables an acidophile to utilize the large pH gradient which exists across the plasma membrane to drive K^+ uptake.

N 109 THE CLONING, EXPRESSION AND SEQUENCING OF THE GALACTOSE/H+ SYMPORT PROTEIN FROM *ESCHERICHIA COLI* : HOMOLOGIES TO MAMMALIAN GLUCOSE AND BACTERIAL SUGARTRANSPORTERS, Paul E. Roberts, Duncan C.M. Moore, Peter J.F. Henderson, Department of Biochemistry, University of Cambridge, Cambridge, CB2 1QW, UK.

The entry of galactose into *E. coli* is mediated by several transport systems, including a galactose/H+ symport protein (GalP). The *galP* gene from *E. coli* was mapped at 3100 kb on the Kohara map (1). A 2·5 kb *Pvu* II fragment thought to contain the gene was cloned into plasmid pBR322 to form plasmid pPER3. This plasmid was found to confer activity characteristic of GalP on a GalP- strain (JM1100) – uptake of [^{3}H]-galactose and a galactose-promoted alkaline pH change. A preparation of membrane proteins from JM1100 containing plasmid pPER3 contained a protein of *Mr* 34 kDa that was absent from controls. This protein is probably GalP. The 2·5 kb *Pvu* II fragment was sequenced. It contained an open reading frame the predicted amino acid sequence of which is about 30% identical to glucose transporters from rat brain, mouse liver and human erythrocyte, heart and adipose tissue and about 70% identical to the arabinose/H+ symporter (AraE) from *E. coli* (2-5). The relative homologies are especially interesting because of the similarity in substrate specificity between GalP and the mammalian glucose transporters and their reaction with cytochalasin B, and the differences in specificity between GalP and AraE. This will help to illuminate their mechanisms of substrate recognition and translocation.

This research is supported by MRC, SERC, the SmithKline Foundation and the Wellcome Trust.

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N 110 IDENTIFICATION AND CHARACTERIZATION OF A UNIQUE PLASMA MEMBRANE H+-ATPase OF D. ACIDOPHILA, Israel Sekler and Uri Pick, Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel. *Dunalliela acidophila* is an unicellular halotolerant green algae that grows optimally at pH 0-1 while maintaining an internal pH of 6.5-7.2. A plasma membrane preparation of this algae has been purified on sucrose density gradients. The preparation has and unusually active vanadate sensitive ATPase of 1-2 μmole/mg/min which is 15-30 fold higher than the activity of a neutrophilic species *D salina.*

The following observations suggest that it is a plasma membrane H+-ATPase:

(1) ATP catalyses proton uptake, demonstrated by fluorescence quenching of an acridine dye (ACMA), and generates a membrane potential, positive inside, which was followed by Oxanol VI absorption changes.

(2) ATP hydrolysis and H+ uptake are inhibited by vanadate, DES, DCCD and erytrosine but not by molybdate azide or nitrate.

(3) ATP hydrolysis and H+ uptake are stimulated by fussicoccin in a pH-dependent manner similar to plant PM H+-ATPases.

(4) Antibodies against yeast PM H+-ATPase cross-react with a 70 KDal polypeptide which is enriched in the membrane preparation. Unusual properties of this enzyme are:

(a) The Km for ATP is around 60 μM, which is considerably lower than in other PM H+-ATPases.

(b) The pH optimum is 6, which is more acidic than for other PM H+-ATPase.

(c) The ATPase activity and H+ uptake are stimulated 350-500% by K+.

These results suggest that *D. acidophila* possesses are unusually active vanadate sensitive H+-ATPase which enables it to sustain the large pH gradient across the cell membrane.

N 111 THE *Salmonella typhimurium mgtB* LOCUS ENCODES A Mg^{2+}-TRANSPORTING P-TYPE ATPase THAT IS HOMOLOGOUS TO EUKARYOTIC Ca^{2+} ATPases AND SUBJECT TO TRANSCRIPTIONAL REGULATION, Marshall D. Snavely and Michael E. Maguire, Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106.

The *mgtB* locus encodes one of three distinct Mg^{2+} transport systems in *S. typhimurium*. The predicted amino acid sequence of MgtB identifies it as an ion-transporting ATPase homologous to the family of P-type ATPases. MgtB shows greater amino acid sequence similarity to the Ca^{2+} ATPases of yeast (PMR1) and mammalian sarcoplasmic reticulum than it does to prokaryotic P-type ATPases. This sequence similarity is particularly apparent in ten highly conserved regions of probable functional significance. Furthermore, comparison of MgtB and other ion transporting ATPases reveals interesting patterns of conservation in six amino acids proposed to be involved in cation binding to the Ca^{2+} ATPase from rabbit sarcoplasmic reticulum (Clarke *et al.* (1989) Nature, 339:476). Based on sequence data and loss of complementation due to insertional inactivation, an accessory protein may be required for MgtB funtion.

Uptake of cation by MgtB is repressed by the presence of divalent cation in the growth medium. Repression is apparent at 20 μM and is maximal above 5 mM. The decrease in ion accumulation is the result of a decrease in the V_{max} of transport without a change in the K_m. These results suggest regulation of transporter number rather than regulation of the properties of existing transporters. Fusions of *mgtB* to *lacZ* place the production of beta-galactosidase under the control of the *mgtB* promoter. Studies using such fusions demonstrate that Mg^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+} inhibit transcription from the *mgtB* promoter when present in the growth medium. Interestingly, only Mg^{2+} and Ni^{2+} are transported by the MgtB system. Transcription regulation by Mg^{2+} is a novel observation, as is transcriptional regulation by Ca^{2+} in a prokaryotic system. (Supported by NIH RO1 GM26340.)

N 112 PROTONMOTIVE FORCE AND DNA-PHAGE TRANSPORT IN STAPHYLOCOCCUS AUREUS, Albert.I.Vinnikov, Sergei P.Kliavzo, Dniepropetrovsk State University, 320625 Dniepropetrovsk, USSR

DNA-phage transport through the cytoplasmic membrane of the bacterial cell is one of the stages of the transduction process. The relationship between the DNA-phage transport and protonmotive force generation in staphylococci was studied.

At the change of the pH value of the ambient medium from 5 to 8 the membrane potential increased from 100 to 160 mV, the value of proton gradient decreasing from 90 to 40 mV. The protonmotive force was within 190-198 mV. Inhibition of protonmotive force generators resulted in lower infection efficiency during phage nucleic acid injection.

Valinomycin and nigericin did not influence separately on staphylococci infection. The joint effect of ionophore antibiotics resulted in lower infection efficiency. This proves the participation of both components of the protonmotive force: transmembrane difference of electric potentials and proton gradient in the phage nucleic acid translocation in staphylococci.

Poster Session-II

N 200 INCREASED LIVER GLUCOSE TRANSPORTER PROTEIN AND mRNA IN INSULIN-DEFICIENT DIABETIC RATS, Tomoichiro Asano, Yoshikazu Shibasaki and Yoshitomo Oka, Third Department of Internal Medicine, University of Tokyo School of Medicine, Hongo, Tokyo, Japan

The effect of insulin-deficient diabetic state on glucose transporter amount and its mRNA level in rat liver have been studied. Rats were injected with 65 mg/kg of streptozocin to become diabetic and were maintained for 10 days and then treated with insulin (insulin-treated diabetic rats) or without insulin (diabetic rats) for following 5 days. Liver glucose transporter with Mr 55,000 was observed to be increased approximately 2-fold in the membranes from liver homogenates of diabetic rats compared to control rats, when assessed by Western blot analysis using antipeptide antibody directed against rat liver glucose transporter. By contrast, no difference in the amount of glucose transporter was observed between control rats and insulin-treated diabetic rats. Liver glucose transporter amount was also increased 10 days after streptozocin injection, indicating that following 5 days of insulin-treatment restored the increased glucose transporter amount to normal level. Northern blot analysis revealed that the alteration in rat liver glucose transporter mRNA was parallel to that in liver glucose transporter protein, suggesting that increased synthesis of liver glucose transporter contributes to increased glucose transporter amount in diabetic rats. These results are different from the results reported in rat adipocytes, indicating that liver type glucose transporter isoform in rat liver is regulated by different mechanisms from muscle/adipocyte type or HepG2 type glucose transporter in rat adipocytes in insulin-deficient diabetic state. Whether or not a similar regulation of liver type glucose transporter isoform is observed in *vitro* will be also discussed.

N 201 IMPORT OF THE IRON-SULFUR PROTEIN OF THE BC-1 COMPLEX INTO YEAST MITOCHONDRIA, Diana S. Beattie, William W. Fu and Shanker Japa, Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506

The gene for the Rieske iron-sulfur protein of the b-c_1 complex was subcloned into the expression vector SP6-4, transcribed and translated in a reticulocyte lysate in the presence of radiolabeled methionine. The time course in vitro of import of this protein into mitochondria at 18^0 revealed the sequential processing of a precursor (p) form to an intermediate (i) and mature (m) form. Both i and m forms were resistant, but the p form was sensitive, to digestion by exogenous proteinase K suggesting that the p form is processed to the i form during translocation into the mitochondrial matrix. Mitoplasts (lacking the outer mitochondrial membrane) could also process the p form to both i and m forms suggesting that both processing events occur in the inner membrane-matrix fraction; however, the m form was then sensitive to digestion by proteinase K suggesting that it has been translocated back across the inner membrane to an external location. Increasing concentrations of the metal chelators EDTA and o-phenanthroline blocked first the conversion of the i to m form and then the p to i form indicating that two different metal-dependent proteases are involved in the 2 step processing of the iron-sulfur protein. At temperatures below 12 ^{o}, the i form accumulated in the matrix fraction and could be chased to the m form at higher temperatures. The m form produced at 12^0 was sensitive to digestion by proteinase K suggesting that proper processing of the i form and the assembly of the m form with other subunits of the b-c_1 complex requires greater fluidity of the inner membrane. Supported by NSF DCB 8716338

N 202 THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER IS ALSO A NUCLEOTIDE BINDING PROTEIN, Anthony Carruthers, Amy L. Helgerson and Daniel N. Hebert, Department of Biochemistry and Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA 01655.

Acute sugar transport regulation is characterized by two basic phenotypes: sensitivity to insulin (Type I, e.g. adipose) and sensitivity to cellular metabolic status (Type II, e.g. nucleated erythrocytes). Skeletal muscle is a mixed phenotype displaying both forms of regulation. While the molecular basis of Type II regulation is unknown, studies with muscle suggest a Type I-*independent* mechanism involving the erythroid-type glucose carrier (GT1). Our studies have revealed a potential mechanism for Type II sugar transport regulation.

GT1 is also a nucleotide binding protein, containing an intracellular site which binds adenosine ribonucleotides with order of affinity ATP > ADP > AMP. ATP binding modifies GT1 higher order structure and reduces carrier *intrinsic* activity (k_{cat}) by 10 to 15-fold. ADP and AMP act as competitive inhibitors of ATP binding and thus of ATP-modulations of carrier structure and activity. These findings suggest that GT1 may be subject to negative feedback control by ATP which is, in turn, amplified by altered cytosolic AMP and ADP levels. These observations may also be relevant to Type II regulation in muscle where anoxia, metabolic depletion and contraction (conditions that elevate sarcoplasmic ADP and AMP) stimulate sugar transport. Simulations of GT1-mediated sugar transport in normoxic and anoxic cardiac muscle support this conclusion.

N 203 HEXOSE TRANSPORT STIMULATION AND MEMBRANE REDISTRIBUTION OF GLUCOSE TRANSPORTER ISOFORMS IN RESPONSE TO CHOLERA TOXIN, DIBUTYRYL CYCLIC AMP AND INSULIN IN 3T3-L1 ADIPOCYTES, Brian M. Clancy and Michael P. Czech, Univ. of Mass. Medical Center, Program in Molecular Medicine, Worcester, MA 01605. Exposure of differentiated 3T3-L1 adipocytes to 100 ng/ml of cholera toxin (CTx) or 1 mM dibutyryl cyclic AMP (Bt_2cAMP) caused a marked stimulation of deoxyglucose transport. A maximal increase of 10 to 15 fold was observed after 12 to 24 h of exposure, while 100 nM insulin elicited an increase of similar magnitude within 30 min. In contrast, neither the B subunit of CTx nor pertussis toxin mimicked the stimulatory effects of these agents on hexose transport. An anti-C-terminal peptide antisera against the glucose transporter isoform present in HepG2 cells and human erythrocytes, and a monoclonal antibody against the muscle and adipocyte glucose transporter isoform were used to detect these transporters in membrane fractions from 3T3-L1 adipocytes. A short term exposure (4 h) of cells to CTx or Bt_2cAMP resulted in a 3 to 4 fold increase in deoxyglucose transport which was associated with the redistribution of both glucose transporter isoforms from low density microsomes to the plasma membrane fraction. However, these changes in the membrane distributions of both transporter proteins were small compared to those observed in cells exposed to insulin for 30 min. Total cellular amounts of both transporter proteins remained constant. In contrast, cells exposed to CTx or Bt_2cAMP for 12 h exhibited elevations in total cellular contents of the HepG2/erythrocyte glucose transporter to about 1.5 and 2.5 fold above controls, respectively. No change in the total cellular amounts of the muscle/adipocyte transporter isoform was observed in cells exposed to these agents for 12 h. Although such treatments of cells with CTx (12 h) versus insulin (30 min) caused similar 10 fold enhancements of deoxyglucose transport, a striking discrepancy was observed with respect to plasma membrane content of glucose transporter proteins. While insulin elicited a 2.6 fold increase in plasma membrane muscle/adipocyte glucose transporter levels, CTx increased the amount of this transporter by only 30% in the plasma membrane fraction. Insulin or CTx increased plasma membrane levels of the HepG2/erythrocyte glucose transporter equally (1.6 fold). Thus, a greater number of glucose transporters in the plasma membrane fraction is associated with transport stimulation by insulin compared to CTx. Based on these data we conclude that: 1) at early times (4 h) after the addition of CTx or Bt_2cAMP to 3T3-L1 adipocytes, redistribution of glucose transporters to the plasma membrane contributes to elevated deoxyglucose uptake rates, and 2) the stimulation of hexose uptake after prolonged treatment (12-18 h) of cells with CTx involves an additional increase in the intrinsic activity of one or both glucose transporter isoforms.

N 204 INCREASED PHOSPHORYLATION OF A CLATHRIN COATED VESICLE ASSOCIATED 120 KDAL POLYPEPTIDE IN RESPONSE TO INSULIN. Silvia Corvera and Renold Capocasale, University of Massacusetts Medical School,Worcester, MA. 01655

Receptor-mediated endocytosis of transferrin, IGF-II, mannose-6-phosphoproteins, and alpha 2 macroglobulin is stimulated by insulin and other growth factors in various cell lines. We recently found that this effect is accompanied by a 3-5 fold increase in the concentration of clathrin heavy chain associated to plasma membranes [Corvera, S (1990) J. Biol. Chem, in press], suggesting that the process of clathrin coated pit/vesicle formation can be regulated by growth factors. We tested the hypothesis that this effect might involve changes in the phosphorylation of proteins associated to clathrin-coated vesicles. Coated vesicles isolated from ^{32}P-phosphate-labeled rat adipocytes were found to contain four phosphopeptides of 32, 85, 95 and 120 Kdal. The 32 Kdal band comigrated with purified clathrin B light chain and was heat stable, suggesting that the clathrin B light chain is phosphorylated in rat adipocytes No consistent changes in the amount of ^{32}P associated to this band or to the 85 and 95 Kdal proteins were detected in response to insulin treatment. However, a 3-5 fold increase in the amount of ^{32}P incorporated into the 120 Kdal band was observed at low concentrations of insulin. This effect was maximal within 5 minutes of stimulation, and persisted as long as the hormone was present. Phosphoaminoacid analysis of an acid hydrolysate of this band revealed that insulin enhanced the phosphorylation of serine, but not threonine or tyrosine residues. Moreover,phorbol esters or cAMP analogues failed to mimic the effect of insulin on the phosphorylation of this protein. This phosphoprotein migrated at a slightly lower molecular weight than ATP-citrate lyase, and was not precipitated by polyclonal antibodies against this enzyme. This novel insulin sensitive phosphoprotein may be important for the process of coated vesicle formation and its regulation by growth factors.

N 205 INHIBITION OF HUMAN ERYTHROCYTE GLUCOSE AND ANION TRANSPORT BY A PHOTOAFFINITY AGENT IS ASSOCIATED WITH CELL SHAPE AND VOLUME CHANGES.
D.F. Diedrich and M.E. Blank, Center of Membrane Sciences and Department of Pharmacology, University of Kentucky, College of Medicine, Lexington, Kentucky 40536.

3-Methoxyglucose and chloride equilibrium exchange are blocked when as few as 1-2 million molecules of p-azidobenzylphlorizin are bound to each RBC. At this same low level, the azide in isosmotic NaCl or KCl media, pH 7.4 phosphate, 20°C, causes 1) an influx of water (max. 10%) but without net Na^+ or K^+ influx, (microhematocrits and atomic absorption spectroscopy), 2) membrane alterations and spicule formation (scanning EM), 3) an initial decrease in 800 nm light scattering by a 0.2% cell suspension, followed by an increased scattering at higher azide levels, 4) no detectable alteration of the order and motion of the lipid bilayer near the outer cell surface (the ESR signal of a lipid-specific spin label that had been co-incorporated into the membrane remained unchanged), and 5) a change in the physical state of the phospholipid/water interface. Effects 1,2 and 3 are complete in 1-2 min after azide addition; the degree of alteration is concentration dependent and remains constant for at least an hour. Cell shape and volume changes elicited by the azide in dim light are fully reversible (halftime = 1.5 min after drug dilution) but become irreversible when the membrane bound ligand is photolyzed and covalently incorporated into membrane components (hemoglobin remains unlabeled). Different effects of the azide are observed at two log units higher concentration, i.e. membrane channels are created that allow salt and water influx; cell swelling occurs, leading to lysis within 10 min depending upon cell storage time, temperature and pH.

N 206 COMPARTMENTATION AND TURNOVER OF THE LOW DENSITY LIPOPROTEIN RECEPTOR IN SKIN FIBROBLASTS, James F. Hare, Dept. of Biochemistry, Oregon Health Sciences University, Portland, OR 97201. Cell surface biotinylation followed by immunoprecipitation of the low density lipoprotein receptor (LDL R) from radiolabeled skin fibroblasts and then isolation of derivatized protein was used to assess receptor protein cellular compartmentation and intercompartmental transfer. 31-49% of LDL R labeled to steady state is derivatized at 4°C, 69-74% at 18°C, and the remaining receptor is derivatized at higher temperatures. At 18°C the amount of LDL R that is derivatized saturates after 1-2 h of reaction showing that access of derivatizing agent to the receptor is limited by temperature and not reaction time. On the basis of these studies and others, only protein exposed to the cell surface reacts at 4°C, while raising the temperature of biotinylation to 18°C provides access to an additional membrane compartment. While total cellular LDL R turns over in a first order manner independent of serum ($T_{½}$ = 12-13 h), the specific activity of pulse-labeled 4°C accessible protein peaks after 1-2 h of chase and reaches a reduced level after 3 h of chase. By contrast, steady state labeled receptor derivatized at 4°C decays at a rate identical to intracellular (underivatized) receptors. These results show that the newly synthesized LDL R is transiently enriched in a 4°C accessible compartment prior to achieving equilibrium distribution between the cell surface and intracellular pools. Thus the steady state distribution of the LDL R between surface accessible and inaccessible plasma membrane compartment(s) is determined immediately after but not before newly synthesized receptor reaches the cell surface.

N 207 INSULIN-REGULATION OF HUMAN GLUT1 AND MOUSE GLUT1 AND GLUT4 GLUCOSE TRANSPORTER ISOFORMS IN MOUSE 3T3-L1 CELLS, Scott A. Harrison, Joanne M. Buxton and Michael P. Czech. Program of Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA 01605. Complementary DNA encoding a HepG2 cell facilitated glucose transporter was subcloned into a mammalian expression vector, pLEN (California Biotechnology). The new construct, pLENGT, contains metal inducible, human metallothionein gene II promoter sequences and the entire coding region of the HepG2 glucose transporter mRNA. Since the HepG2 glucose transporter protein is not regulated by insulin in the hepatoma cells, we utilized pLENGT to study insulin regulation of human HepG2 transporter in insulin-responsive mouse 3T3-L1 cells. Additionally, nontransfected mouse 3T3-L1 adipocytes express HepG2 type (GLUT1) and insulin-regulated skeletal muscle/adipocyte type (GLUT4) glucose transporters. Therefore, we utilized this model system to contrast insulin-regulation of GLUT1 and GLUT4 proteins. Mouse 3T3-L1 fibroblasts were transfected with pLENGT and subclones were obtained which exhibited zinc inducible expression of human GLUT1 mRNA, protein and glucose transport activity, before and after differentiation into adipocytes. Human and mouse GLUT1 proteins were expressed in both the mouse preadipocytes and adipocytes. The GLUT4 protein was only detected in differentiated 3T3-L1 cell lines. Insulin treatment stimulated sugar uptake in control preadipocytes 73%, in the presence or absence of 75 μM zinc. In contrast, preadipocytes expressing human GLUT1 protein exhibited 2-3 fold elevated basal sugar uptake, while insulin-stimulated uptake decreased to 47% and 27%, respectively, in the absence or presence of zinc. Treatment of control adipocytes with 75 μM zinc had no effect on basal or insulin-stimulated sugar uptake. Zinc treatment of pLENGT adipocytes caused a 2 fold increase in basal 2-deoxyglucose uptake which was additive with the insulin-stimulated increase in the rate of sugar uptake. Mouse GLUT1 and GLUT4 and the constituitively expressed human GLUT1 proteins were redistributed from low density microsomal membrane to plasma membrane fractions in response to insulin. In zinc treated pLENGT adipocytes where basal transport was elevated 2 fold, the cells were fully responsive to insulin with respect to sugar transport and GLUT4 protein movement to the plasma membrane, but insulin did not elicit an additional increase in GLUT1 protein in the plasma membrane fraction. These observations demonstrate that membrane distributions of a glucose transporter protein which is not responsive to insulin in HepG2 cells and two mouse glucose transporter isoforms, are regulated by insulin when expressed in mouse 3T3-L1 adipocytes. However, in spite of the clear insulin regulation of transporter membrane distribution, expressed human GLUT1 appears to contribute significantly only to the rate of basal uptake and not to the insulin-stimulated increase in the rate of 2-deoxyglucose uptake in 3T3-L1 adipocytes.

N 208 ESSENTIAL ROLE FOR Ca^{2+} INFLUX FOLLOWED BY $[Ca^{2+}]_i$ RISE IN THE MATING PHEROMONE RESPONSE PATHWAY OF THE YEAST SACCHAROMYCES CEREVISIAE. Hidetoshi Iida and Yasuhiro Anraku, Division of Cell Proliferation, National Institute for Basic Biology, Okazaki 444, Japan.
A mating pheromone of the yeast Saccharomyces cerevisiae, α-factor, is known to induce Ca^{2+} influx in cells of a mating type. However, the role of intracelluar Ca^{2+} remains unknown in this organism. We investigated changes in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual cells in response to α-factor using fura-2 as a Ca^{2+}-specific probe in conjunction with digital image processing. The addition of α-factor to a cells raised $[Ca^{2+}]_i$ to several hundred nanomolar in the cells from a basal level of approx. one hundred nanomolar, simultaneous with the induction of Ca^{2+} influx. When the cells were incubated with α-factor in a Ca^{2+}-deficient medium, Ca^{2+} influx was greatly reduced and the rise in $[Ca^{2+}]_i$ was not detected. In this condition, the cells died specifically after they had changed into cells with one projection on the cell surface. This indicates that the rise in $[Ca^{2+}]_i$ is essential for the late response to α-factor. The duration of Ca^{2+} requirement for maintaining viability was limited to this stage and the earlier and later stages were not affected by Ca^{2+} deprivation. Mating between a and α cells was impaired in this medium due to cell death at and before the stage of conjugation.

N 209 **ATP Dependent Dissociation from GRP78 is Essential for Secretion of Some Heterologous Proteins in Chinese Hamster Ovary Cells. 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), are efficiently secreted and exhibit a slight transient association with GRP78, a resident luminal protein of the ER. In contrast, proteins which are inefficiently secreted, factor VIII and tPA-3X (a mutant which lacks the 3 N-linked glycosylation sites), exhibit a stable association with GRP78. We have analyzed the importance for ATP availability in secretion of these proteins by studying the effect of ATP depletion by addition of CCCP, an uncoupler of oxidative phosphorylation. Low concentrations of CCCP inhibited secretion of factor VIII but not vWF synthesized in the same cell. As the concentration of CCCP increased, secretion of vWF was also inhibited. In the presence of low concentrations of CCCP, the majority of the factor VIII was retained a stable complex with GRP78. Low concentrations of CCCP also inhibited the secretion of tPA-3X, but not wildtype tPA. These studies demonstrate that proteins associated with GRP78 require ATP as a necessary step in their transport from the endoplasmic reticulum and we speculate that available ATP levels may be partly responsible for reduced secretion efficiency.

N 210 A CA^{2+}-ACTIVATED K^+ CURRENT IN CORN PROTOPLASTS MAY REGULATE ELECTRICAL HOMEOSTASIS FOLLOWING STIMULUS-INDUCED CA^{2+} INFLUX. Karen A. Ketchum and Ronald J. Poole Dept. of Biology, McGill Univ., 1205 Dr. Penfield Avenue, Montreal, PQ H3A 1B1 Canada
Transient depolarization of the membrane potential in plant cells is associated with signal transduction pathways initiated by both hormonal and environmental stimuli. We have been interested in identifying the ionic currents which mediate these shifts in potential and the cellular processes which regulate their activity. Here we present evidence that the voltage- and time-dependent K^+ current (I_{K+}), evoked by depolarization of corn protoplasts, is also activated by cytosolic Ca^{2+}. The addition of organic Ca^{2+} channel blockers from the dihydropyridine (nitrendipine) and phenylalkylamine (verapamil, D600) families caused marked inhibition of I_{K+}. The IC_{50} for nitrendipine (concentration which produced 50% reduction in current) was 1 μM at a test potential of +60 mV following a 20 minute incubation period. These data suggested that Ca^{2+} influx was necessary for I_{K+} activation. We tested this premise further by manipulating either the Ca^{2+} buffering capacity or the free Ca^{2+} concentration of the intracellular medium (pipette filling solution). When the EGTA concentration was increased from 4 to 40 mM, thus preventing changes in cytosolic Ca^{2+}, I_{K+} was reduced by 72%. In contrast, raising the free Ca^{2+} concentration from 30 to 300 nM, with a constant EGTA level, caused a 2-fold stimulation of I_{K+}. These results provide evidence for Ca^{2+}- and voltage-activated K^+ channels at the plasma membrane of plant cells. Current through these channels would repolarize the membrane during signalling events which combine membrane depolarization and changes in cytosolic free Ca^{2+} concentration.

N 211 SUBCELLULAR DISTRIBUTION OF GLUCOSE TRANSPORTERS IN SKELETAL MUSCLE: EFFECTS OF INSULIN, EXERCISE AND DIABETES. Amira Klip, Toolsie Ramlal, Andre Douen, Dimitrios Dimitrakoudis, Philip Bilan, Gregory Cartee, John Holloszy & Mladen Vranic. The Hospital for Sick Children, Toronto, Canada M5G 1X8.

The cellular basis of insulin and exercise action in muscle and the cause of insulin resistance in diabetes were investigated. Insulin and acute exercise stimulated glucose uptake in rat hindquarter muscles by 5- and 3-fold, respectively. Subcellular fractionation of hindquarter muscles yielded plasma membrane (PM) and intracellular membrane (IM) fractions. The number of glucose transporters (GTs) was assessed by D-glucose-protectable binding of cytochalasin B (CB), and the specific isoforms GLUT-1 and GLUT-4 were detected immunologically in Western blots. In the basal state, GLUT-1 was primarily in the PM, and GLUT-4 was in both IM and PM (more abundant in IM). Insulin decreased CB binding and GLUT-4 content in IM by ~50% and increased CB binding and GLUT-4 in PM ~2-fold. Acute exercise did not affect IM but doubled the CB binding and GLUT-4 content of the PM. The distribution of GLUT-1 was not altered by either insulin or exercise.

Rats made diabetic by streptozocin injection were normoinsulinemic but hyperglycemic after 7 days. Basal glucose uptake into hindquarter muscles of diabetic rats was reduced by 71%, and maximally insulin-stimulated glucose uptake was reduced by 48%. CB binding decreased by 42% in the PM and 30% in IM. GLUT-4 was also reduced in muscle homogenates, PM and IM of diabetic muscle. The reduction of GTs in the PM may account for the diminished rate of basal glucose uptake in diabetic muscle. Normalizing glycemia with phlorizin resulted in recovery of normal CB binding levels. In diabetic hyperglycemic and control rats, insulin perfusion decreased CB binding in IM to similar extents (0.69 and 0.73 pmol/mg, respectively); however, the insulin-dependent gain in GTs in the PM was lower in diabetic than controls (0.86 and 1.32 pmol/mg). The GLUT-4 content paralleled these changes. It is proposed that less insulin-dependent recruitment of GTs to the plasma membrane, compounded by fewer basal GTs, accounts for the insulin-resistance of glucose uptake in skeletal muscle of diabetic rats.

N 212 **THE *Saccharomyces cerevisiae HXT2* GENE ENCODES A SUGAR TRANSPORTER HOMOLOGOUS TO A FAMILY OF PROCARYOTIC AND EUCARYOTIC SUGAR TRANSPORTERS.** *Arthur L. Kruckeberg and Linda F. Bisson, **Department of Viticulture and Enology, University of California, Davis, CA 95616.***

The *HXT2* gene of the yeast *Saccharomyces cerevisiae* encodes a protein which imparts high affinity glucose transport to yeast cells. Genetic analysis localized *HXT2* to a 2.9 kb DNA fragment. The sequence of this fragment revealed a single long open reading frame of 1623 base pairs. The predicted protein product of 541 amino acids is highly homologous to a family of sugar transporters from bacteria, yeast, and mammals. It is 28% identical and 54% similar to the human erythrocyte glucose transporter. The two proteins display very similar hydropathy profiles, suggesting that they are both comprised of twelve transmembrane domains, interrupted between domains six and seven by a long hydrophilic, cytoplasmic domain. The HXT2 protein has the same *N*-linked glycosylation site as found in the erythrocyte transporter (NQT) at the homologous position in the secondary structure. Southern blot analysis of yeast genomic DNA demonstrated the existence of a number of related sequences in yeast; however, *HXT2* is present at only a single copy per genome. The levels of *HXT2* mRNA are affected by the carbon source of the yeast medium.

N 213 FUNCTIONAL CHARACTERIZATION OF THE INTESTINAL cDNA CLONE GLUT5 FOR D-GLUCOSE TRANSPORT.

E.Brot-Laroche, L. Mahraoui & G.I. Bell. Unité 178 de l'INSERM, 16 ave P. Vaillant Couturier, 94807 Villejuif, France & Howard Hughes Medical Institute Research Laboratories, Department of Biochemistry & Molecular Biology and Medecine, University of Chicago Hospitals, 5841 S. Maryland Avenue, N237A Box391 Chicago, Illinois 60637

D-glucose transport across the membranes of enterocytes is mediated by three independent transporters: the Na/D-glucose cotransporter (BBS1) and a low affinity, high capacity carrier (BBS2, ref) are located in the brush border membrane, the third one being in the basolateral membrane. We report here results showing the expression in Xenopus laevis oocytes, of an human intestinal cDNA-glucose-carrier clone, GLUT5. This cDNA encodes for a stereospecific D-glucose, D-galactose and 2 deoxy-D-glucose transporter sensitive to cytochalasin B. However glucose influx was not affected by phlorizin nor by phloretin, the known inhibitors of respectively the cotransporter and the basolateral carrier. GLUT5 is Na-independent and exhibits a low affinity (30 mM) for monosaccharide transport. These characteristics are similar to those reported for BBS2 and already suggest that GLUT 5 and BBS2 could be identical.

E. Brot-Laroche, M. A. Serrano, B. Delhomme & F. Alvarado. 1986, J. Biol. Chem 261, 6168-6176

N 214 RAPID REGULATION OF GLUCOSE TRANSPORTER BY NUTRITIONAL-HORMONAL STATUS.

A. Leturque, C. Postic, P. Ferré, D. Perdereau, C. Coupé, J. Girard. Centre de Recherche sur la Nutrition, 9 rue Jules Hetzel 92190 Meudon Bellevue France.

In adipose tissue and muscles of suckling newborn rats, glucose utilization rate (transport and metabolism) is low and insensitive to insulin. The factors involved in the increase in insulin response observed at weaning have been analyzed. The hormonal status of the newborn rat is modified rapidly by nutritional means. Weaning the rats on a high-carbohydrate diet induces an increased plasma insulin which is prevented by weaning on a high-fat diet. A three-fold increase in both the amount of mRNA encoding for the insulin responsive glucose transporter (IRGT) and the glucose transporter protein itself is induced by weaning the rats on the adult high-carbohydrate diet. In contrast, weaning on a high-fat diet has no effect. Six hours after feeding a single high-carbohyrate meal to suckling rats, an increase in IRGT transcripts is observed in adipose tissue. Similar effects of mRNA levels are observed for 2 lipogenic enzymes, fatty acid synthetase and acetyl CoA carboxylase. This suggests that the nutritional-hormonal environment controls rapidly the expression of factors involved in insulin action and specially IRGT.

N 215 THE MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF A GENE, *HXT1*, INVOLVED IN HIGH AFFINITY, KINASE-DEPENDENT HEXOSE TRANSPORT IN *SACCHAROMYCES CEREVISIAE*. **Deborah A. Lewis and Linda F. Bisson., Department of Viticulture and Enology, University of California, Davis, CA. 95616.**

Yeast have at least two hexose transport systems: constitutive low-affinity transport (Km=10-20 mM) and glucose repressible, kinase-dependent high-affinity transport (Km=1-2 mM). A null mutation in the *SNF3* (sucrose-non-fermenting) locus results in a loss of high affinity glucose transport. Several non-allelic genes were isolated which complemented the *snf3* null. The gene *HXT1* was obtained in a multicopy plasmid (pSC7) and was found to relieve the growth defect of the high affinity transport mutant, *snf3::HIS.HXT1* in multicopy but not in single copy fully restored kinase-dependent, high-affinity hexose transport to the *snf3* null. Sequence analysis of the *HXT1* gene has revealed an open reading frame encoding a protein of at least 526 amino acids in length and analysis of the hydrophobicity profile suggests that the *HXT1* protein contains 12 putative membrane-spanning regions similar to other hexose transporters in yeast, bacteria and mammals. Southern blot analysis of several yeast strain genomes using a *HXT1* probe at high stringency showed the gene to be in single copy and at low stringency has revealed a family of cross-hybrizing fragments. *HXT1* gene disruption analysis will allow the assessment of essentiality and the mutant phenotype effects on glucose transport and its regulation.

N 216 THE PRESENCE OF TWO TRANSCRIPTS FOR THE HEXOSE TRANSPORTERS IN UNDIFFERENTIATED RAT MYOBLASTS, Theodore C.Y. Lo, Patricia Kudo, Thomas Kovala, Ling Xia, and Hari R. Singal, Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1.

We have recently demonstrated the presence of a high-affinity and a low-affinity hexose transport system (HAHT and LAHT, respectively) in undifferentiated rat myoblast L6. These systems differ in their substrate specificity, sensitivity to various inhibitors, cytochalasin B binding properties, and in their response to glucose-starvation. HAHT also differs from LAHT in that it cannot be detected in multinucleated myotubes. Mutants defective only in HAHT, but not in LAHT, have been isolated and characterized. Using a HepG2 glucose transporter cDNA probe (kindly supplied to us by Dr. G. Bell), a 1.8 kb and a 2.8 kb transcript were detected in undifferentiated L6 myoblasts in Northern gel blotting studies. The 1.8 kb transcript could not be detected in HAHT$^-$ mutants containing only residual amount of the HAHT transporter. This suggests that the 1.8 kb transcript may be encoding for the HAHT transporter. The 1.8 kb transcript was also present in much reduced level in multinucleated myotubes, thus suggesting that the HAHT transporter was not synthesized in myotubes. Similar to the LAHT activity, the level of the 2.8 kb transcript was found to remain relatively constant in various HAHT$^-$ mutants. Similar to findings by other workers, only the 2.8 kb transcript can be detected in the multinucleated myotubes. This together with the finding that only LAHT activity could be detected in the myotubes suggested that the 2.8 kb transcript might be coding for the LAHT transporter. In summary, the present investigation identifies the transcripts responsible for the hexose transporters present in undifferentiated myoblasts. The identity of these transcripts was confirmed through the use of appropriate hexose transport mutants, and multinucleated myotubes. (Supported by operating grants from the Medical Research Council of Canada to T.C.Y.L.)

N 217 RABBIT INTESTINAL Na^+/GLUCOSE COTRANSPORTER AND RENAL cDNAs ARE HOMOLOGOUS. Ana M. Pajor and Ernest M. Wright. Dept. of Physiology, UCLA School of Medicine, Los Angeles, CA, 90024. The cDNAs encoding the intestinal Na^+/glucose cotransporter have been isolated, sequenced and expressed. Together with a bacterial Na^+/proline cotransporter, putP, they appear to constitute a distinct class of proteins based on sequence homology and function. The renal Na^+/glucose cotransporter may also belong in this class since renal mRNA hybridizes (major band 2.4 Kb) with the cDNA encoding the rabbit intestinal Na^+/glucose cotransporter. Injection of rabbit renal cortex mRNA into Xenopus oocytes results in the expression of Na^+-dependent glucose transport. A cDNA library was prepared from size-fractionated rabbit renal cortex mRNA in the plasmid vector pcDNAII, and screened with the rabbit intestinal clone. Two partial-length clones, encoding 1.6 and 1.0 Kb of the 3' end, were isolated and appear to be highly homologous to the rabbit intestinal clone, based on restriction mapping and sequencing. The longest of these clones (RK52) was used to screen a rabbit renal lambda zap library. Twelve positive clones were purified, ranging in size from 1.5 to 2.5 Kb. Partial restriction maps, which differ from maps of the rabbit intestinal clone and RK52, indicate that these clones fall into at least 4 classes. Slot blot experiments indicate that the renal clones share a lower sequence homology with the intestinal Na^+/glucose cotransporter cDNA and with RK52. Sequencing and functional expression experiments are currently in progress.

N 218 A NEW ESCHERICHIA COLI OPEN READING FRAME CODING FOR A PUTATIVE TRANSPORTER, Wongi Seol, Dashou Wang and Aaron J. Shatkin, Center for Advanced Biotechnology and Medicine, Piscataway, NJ 08854

A previously unrecognized open reading frame (ORF) was found by sequencing a 9 kb cloned fragment of E. coli genomic DNA. The ORF is located between the 3' ends of ribosomal operon rrnG and the gene for phosphatidylserine synthase (pss) in a head-to-head orientation with respect to pss. The amino acid sequence deduced for the new ORF suggests that it encodes a transporter protein. It is 58% similar to the citA citrate transporter that is specified by the naturally occurring plasmid pWR60. Like citA, the E. coli arabinose and xylose transporters and mammalian glucose carrier protein, the deduced sequence of the new ORF includes twelve hydrophobic putative transmembrane domains with duplicated motifs at the third and ninth of these domains. Consistent with its hydrophobic composition, the protein expressed in an in vitro coupled system from a 1.99 kb subclone in SDS-polyacrylamide gels migrates broadly with an apparent molecular weight of ca. 35 kDa, i.e. considerably below the calculated value of 59 kDa. In vivo expression studies are being done to identify a ligand for the putative transporter.

N 219 HORMONE-INDUCED ELEVATION OF cGMP LEVELS IN ISOLATED RAT CARDIOMYOCYTES - A POTENTIAL PATHWAY FOR MODULATION OF GLUCOSE TRANSPORT, Michael F. Shanahan, Edward W. Lam and Barbara A. Guebert, Department of Physiology, Southern Illinois University School of Medicine, Carbondale, IL 62901

Glucose transport in isolated rat cardiomyocytes is stimulated by insulin and catecholamines approximately 2-3 fold over basal rates. We have previously shown that agents which increase cellular cGMP levels exhibit an insulinomimetic effect on 3-O-methylglucose transport in these cells (Endocrinology **125**,1074-1081,1989). We have now extended our characterization of changes in cardiomyocyte cGMP levels and its relation to stimulation of glucose transport. Following the addition of insulin (100 nM) cell cGMP levels increased 2 to 5-fold above basal levels within one minute. This was followed by a gradual decline to 50-100% above basal by three minutes, which was maintained for up to 15 min. The initial rapid transient increase in cGMP preceded activation of glucose transport. Measurement of cGMP levels in the presence of several nucleotide phosphodiesterase inhibitors indicates that the mechanism of this insulin action is via stimulation of guanylate cyclase. Treatment of cells with several cGMP-elevating hormonal agents resulted in stimulation of glucose transport, while agents known to be guanylate cyclase inhibitors (methylene blue and LY83583) lowered cGMP levels and blocked insulin-stimulated transport in a dose-dependent manner. Additional studies are currently being performed using antibodies for the insulin-regulatable glucose transporter (IRGT)(mAb1F8) and HepG2/erythroid GT to compare relative changes in distribution of the GT under the experimental conditions described above.

N 220 IN VIVO REGULATION OF THE ADIPOSE TISSUE INSULIN-RESPONSIVE GLUCOSE TRANSPORTER mRNA BY INSULIN. WI Sivitz, T Kayano, GI Bell and JE Pessin, Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa.

We and others have recently demonstrated that insulin induces a dramatic increase in the *in vivo* expression of the major insulin sensitive glucose transporter (GLUT-4) mRNA in rat epididymal adipose tissue of streptozotocin (STZ)-diabetic rats. We have now determined the chronic effects of insulin therapy and in addition evaluated the effect of insulin vs. altered glycemia and nutrition on the levels of GLUT-4 mRNA.

STZ-diabetic rats demonstrated a specific decrease (~10 fold) in adipose tissue GLUT-4 mRNA. Treatment of STZ-diabetic rats with twice daily injections of insulin for 1-3 days resulted in a ~16 fold increase in the relative amount of GLUT-4 mRNA to levels approximately 2 fold greater than those observed in control animals. However, following 7 days of insulin therapy the amount of GLUT-4 mRNA decreased approximately 2 fold back to the levels found in the control animals. Normalization of the STZ-induced serum hyperglycemia by phlorizin treatment, which inhibits renal tubular reabsorption of glucose, had no effect on GLUT-4 mRNA in the absence of insulin. Further, phlorizin administration did not alter GLUT-4 mRNA levels in adipose tissue of normal rats or prevent the insulin-induction of GLUT-4 mRNA in adipose tissue of STZ-diabetic rats. Similar to STZ-diabetes, fasting for 48 h also reduced adipose GLUT-4 mRNA levels. Parenteral administration of insulin plus glucose over 7.5 h to fasted animals, but not glucose alone, increased the levels of the GLUT-4 mRNA 3 to 4-fold. In contrast to the changes observed in GLUT-4 mRNA, the expression of the rat brain/HepG2 glucose transporter and B-actin mRNA was not significantly altered under the conditions of the above experiments. These data demonstrate that long-term insulin treatment of STZ-diabetic rats results in an initial (3-7) day transient hyperresponsive increase in adipose tissue GLUT-4 mRNA which subsequently declines to normal steady state levels. Further, the relative glycemic state does not influence the GLUT-4 glucose transporter mRNA expression *in vivo* and strongly suggests that insulin is a major factor regulating the levels of GLUT-4 mRNA in adipose tissue.

N 221 CTAP III INCREASES CARBOHYDRATE METABOLISM IN CULTURED FIBROBLASTS BY INCREASING THE NUMBER OF GLUCOSE TRANSPORTERS. P.-K. K. Tai, J.-F. Liao, C. W. Castor, and C. Carter-Su, Depts. Physiology and Internal Medicine, Univ. Michigan Medical School, Ann Arbor, MI 48109. Connective tissue activating peptide III (CTAP III) is a 9 kDa platelet-derived peptide with several smaller forms which exists in high abundance (30-50 ng/ml) in human plasma. By sequence, it belongs to a family of peptides that includes neutrophil activating peptide I (NAP I/interleukin 8), PF-4, MGSA and growth-related proteins (GRO and 9E3/pCEF-4) in transformed cells. Plasma levels of CTAP III are elevated in many patients with rheumatoid arthritis; in clotted blood levels range from 15-35 ug/ml. We shown previously that in cultured human synovial cells, CTAP III has a variety of anabolic effects, including increased glucose transport and synthesis of glycosaminoglycans, hyaluronic acid, prostaglandins and DNA. To gain insight into the molecular mechanisms by which CTAP III regulates carbohydrate metabolism, we have studied the ability of CTAP III to stimulate glucose uptake in 3T3-F442A fibroblasts. In actively growing, but not confluent, fibroblasts or differentiated 3T3-F442A adipocytes, platelet-derived CTAP III (1-30 ug/ml) stimulates uptake of 2-deoxyglucose and 3-O-methyl glucose by 2-5 fold after 16-24 h. Immunoblot analysis, using a polyclonal antibody which recognizes the Hep G2/rat brain/erythrocyte type glucose transporter, indicates that CTAP III increases the total cellular number of glucose transporters by 2-5 fold. The increase in transporter number correlates with the increase in transport in dose response curves. These data suggest that CTAP III and/or its isoforms increase carbohydrate metabolism in growing fibroblasts by increasing the number of cellular glucose transporters.

N 222 EXTRACELLULAR CALCIUM REGULATES TRANSPORT AND DISTRIBUTION OF PROTEOGLYCANS IN A RAT PARATHYROID CELL LINE, Yasuhiro Takeuchi, Masaki Yanagishita, Kazushige Sakaguchi* and Vincent Hascall, Bone Research Branch, NIDR and *Metabolic Diseases Branch, NIDDK, NIH, Bethesda, MD 20892.

A rat parathyroid cell line, which retains differentiated characteristics of the parathyroid gland, was used to study the transport and distribution of heparan sulfate proteoglycans (HSPGs). In this cell line, extracellular calcium concentration ($[Ca^{2+}]_e$) is the major modulator of cell functions. HSPGs were metabolically labeled with [^{35}S]sulfate and their cellular distribution was determined by trypsin-treatment. In steady state, ~70% of the labeled HSPGs are accessible to trypsin in 0.05 mM $[Ca^{2+}]_e$ (low Ca) whereas only ~15% are accessible in 2.1 mM $[Ca^{2+}]_e$ (high Ca). HSPGs labeled for 10 min subsequently distribute during the chase in response to $[Ca^{2+}]_e$. In low Ca, ~60% of the newly synthesized HSPGs reach the cell surface and recycle between endosomes and the cell surface ($t_{1/2}$ ~ 2 min) before their degradation 2 h after labeling. In high Ca, only ~10% of the HSPGs reach the cell surface and they do not recycle. Cells quickly respond to changes of $[Ca^{2+}]_e$ by redistributing labeled HSPGs; HSPGs labeled in high Ca rapidly redistribute to the cell surface and the recycling compartment after extracellular calcium is chelated with EGTA, and vice versa; HSPGs labeled in low Ca rapidly become sequestered in the cells when calcium is added to the medium. Other divalent cations fail to mimic the response to Ca. These observations suggest that $[Ca^{2+}]_e$ specifically regulates the transport, distribution and recycling of HSPGs in a rat parathyroid cell line, and that for these cells a majority of the HSPGs are located in the membrane compartment involved in regulated transport between endosomes and the cell surface.

N 223 SUCROSE TRANSPORT INTO STALK TISSUE OF SUGARCANE, M. Thom and A. Maretzki, Department of Genetics and Pathology, Hawaiian Sugar Planters' Association, Aiea, HI 96701.
The productivity of higher plants is, in part, dependent upon transport of photosynthate (usually sucrose) from "source" (leaves) to "sink" (in sugarcane, stalk) and upon its assimilation in cells of the stalk tissue. Several pathways are possible, including a route of sucrose moving directly from phloem into cells of the storage parenchyma via plasmadesmata connections and another route via transfer of sucrose into the apoplast, followed by uptake across the plasma membrane (pm) of the parenchyma cells. A major point that has been readdressed is whether hydrolysis of extracellular sucrose to hexoses is obligatory for sugar uptake across the pm into cells of the storage parenchyma, whether only sucrose is taken up, or whether both sucrose and hexoses have carrier sites on this membrane. In sugarcane, sucrose had been reported (Sacher, JA, MD Hatch & KT Glasziou 1963 Plant Physiol. 38:348-354) to undergo hydrolysis in the apoplast before uptake into storage parenchyma, whereas recently sucrose was shown to be taken up intact, and only intact (Lingle, S 1989 Plant Physol. 91:6-8) based on lack of randomization of ^{14}C fructosyl labeled sucrose accumulated after feeding tissue slices with this sugar. In this report, we present evidence from slices of stalk tissue that sucrose can be taken up intact via a carrier-mediated, energy-dependent process. The evidence includes: (1) uptake of fluorosucrose, an analog of sucrose not subject to hydrolysis by invertase; (2) little or no randomization of (^{14}C) fructosyl labeled sucrose taken up; (3) the presence of a saturable as well as a linear component of sucrose uptake; and (4) inhibition of sucrose uptake by protonophores and sulhydryl agents. Hexoses can also be taken up, and at a greater efficiency than sucrose. It is probable that both hexoses and sucrose can be transported across the plasma membrane, depending on the physiological status of the plant.

N 224 THE LIVER/BETA CELL GLUCOSE TRANSPORTER IN ISLETS OF NORMAL AND DIABETIC RATS, Bernard Thorens, Suzanne Bonner-Weir, Jack Leahy, Gordon Weir, Harvey F. Lodish, Whitehead Institute, Cambridge, MA 02142 and Joslin Clinic, Boston, MA 02139. The liver/beta cell glucose transporter is expressed in liver, intestine, kidney and pancreatic islets. By immunocytochemistry at the light and electron microscope level, we have shown that in islets it is expressed only by beta cells and that it is preferentially associated with microvilli present on the lateral membrane of these cells. This liver/beta cell transporter has a high K_M for glucose (15-20 mM) and we postulated that it may be part of the glucose sensor of beta cells. In the present study we analysed the level of expression of this transporter in islets of rat models of type II diabetes (neonatal streptozotocin rats, pancreatectomized rats). The beta cells of these rats are characterized by a selective defect in glucose responsiveness while they are still sensitive to other stimuli like arginine. We found both by Western blotting and immunofluorescence analysis that the expression of the liver/beta cell transporter was strongly reduced. This suggests that a reduction in the expression of this transporter may be important to determine the glucose unresponsiveness of type II diabetic islets.

N 225 A GTPγS-SENSITIVE COMPONENT IS INVOLVED IN ENDOCYTIC VESICLE FUSION, Marianne Wessling-Resnick and William A. Braell, Department of Biological Chemistry & Molecular Pharmacology, Harvard Medical School, Boston, MA 02115
We have developed an *in vitro* assay for the early vesicle fusion event of the endocytic pathway based on the association of a fluid phase marker, avidin-β-galactosidase, and the receptor-associated probe, biotin-transferrin. The cell-free reaction not only requires ATP, but is also dependent on the presence of a factor which is sensitive to alkylation by N-ethylmaleimide (NEM) and which is found in cytosol. We have found that vesicle fusion is also inhibited by GTPγS, a non-hydrolyzable analog of GTP, with an apparent EC_{50} of about 1 μM. The GTPγS sensitive component is also found to be a cytosolic factor, since the assay is inhibited by the addition of excess cytosol which has been preincubated at 37°C in the presence of GTPγS. When cytosol is added to membranes which have been pre-treated with NEM, we find that preincubation at 37° C in the absence of GTPγS helps to overcome the inhibitory effect exerted by its presence, suggesting that the GTPγS-sensitive step of the fusion reaction mechanism occurs prior to the stage involving the NEM-sensitive factor. The GTPγS-sensitive activity appears to influence components on the membrane surface itself, rather than cytosolic factors involved in the fusion reaction. Further characterization has shown that other non-hydrolyzable analogs, such as GMP-PNP and GMP-PCP, also inhibit *in vitro* vesicle fusion, as well as ATPγS. Thus, the hydrolysis of both GTP and ATP is required for initial events of the endocytic pathway. (This work is funded by NIH GM34635. MWR is supported by a Damon-Runyon - Walter Winchell Cancer Fund Fellowship, DRG-975).

N 226 DELETION OF C-TERMINAL DOMAIN LOCKS GLUCOSE TRANSPORTER INTO INWARD FACING CONFORMATION AND ABOLISHES ITS ACTIVITY. Yoshitomo Oka, Tomoichiro Asano and Yoshikazu Shibasaki, Third Department of Internal Medicine, University of Tokyo School of Medicine, Hongo, Tokyo, Japan

Recent cDNA cloning has demonstrated that the facilitated glucose transporters (GTs) comprise a family of protein with a very similar structure. In order to understand the structure and function relationship in the facilitated GTs, we have engineered rabbit HepG2 type GT cDNA so that most (37 out of 42 amino acids) of the intracellular C-terminal domain is deleted. This cDNA was subcloned into expression vector designated pMTHneo and transfected into Chinese hamster ovary (CHO) cells (1). Stable overexpression of this deletion mutant into CHO cells was demonstrated by Western blotting with antipeptide antibody against mid-portion of glucose transporter but not with antibody against C-terminal domain (2). The molecular weight difference of 4,000 between the normal and mutated GT corresponds to deletion of 37 amino acids. Very interestingly, no significant increase in transport activity was observed in CHO cells with mutated GT, although a large amount of the mutated GT was demonstrated to be inserted into the plasma membranes by surface labeling method. In contrast, a remarkable increase (8-fold) in transport activity was observed in CHO cells with a similar amount of the expressed normal GT and the activity of mutated GT was estimated to be at most 15 % of that of normal GT. Although the mutated GT was photoaffinity labeled with cytochalasin B (CB) in a D-glucose inhibitable manner, ethylideneglucose, which bind to outer glucose binding site of GT, did not inhibit CB labeling, whereas propyl glucoside, which binds to inner binding site, inhibited CB labeling. In addition, the mutated GT was hardly labeled with photolabel probe ATB-BMPA (a gift from Dr. G. D. Holman), which binds to outer glucose binding site. These results further support an alternating conformation model for the mechanisms of glucose transport. C-terminal domain is not involved for glucose binding to inner binding site but rather needed for having outward facing conformation. Thus, the mutated GT cannot alternate its conformation and is locked into inward facing form, resulting in that glucose is no more able to get through the GT (see schema).

1. Asano, T. et al. J. Biol. Chem. 264, 3416-3420 (1989)
2. Oka, Y. et al. J. Biol. Chem. 263, 13432-13439 (1988)