GENETIC AND IN VITRO ANALYSIS OF CELL COMPARTMENTALIZATION

Organizer: David Meyer

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Keynote Address (Joint)

H 001 PROTEIN FOLDING AND OLIGOMERIZATION IN THE ENDOPLASMIC RETICULUM. Ari Helenius, Ineke Braakman, Utpal Tatu,
Thorsten Marquardt, Craig Hammond, Jonne Helenius, Dept. of Cell Biology, Yale School of Medicine, New Haven, Ct 06510.

The conformational maturation of glycoproteins in the ER, determines how efficiently, how fast an in which form proteins are secreted, expressed on the plasma membrane or delivered to the membranes or lumen of vacuolar organelles. Maturation intermediates, misfolded proteins or misassembled oligomers are generally retained in the ER where they are degraded. In addition to providing a mechanism for restricting the potential damage caused by the deployment of defective proteins, this system is used by the cell to post-translationally regulate expression levels of specific proteins.

In our work, we are trying to define the characteristics that make the ER lumen an efficient folding and sorting environment for a multitude of important proteins. Using mainly viral glycoproteins(Influenza HA and VSV G protein) as model proteins, we study the folding, maturation and 'quality control' processes in living cells. Since these proteins, like most others made in the ER, depend on the formation of disulfides for proper folding, we can—using the pulse-chase approach —follow folding by monitoring the state of oxidation. The folding and oligomerization process can also be analyzed using monoclonal antibodies to conformation dependent epitopes, by morphological methods, by cell fractionation and by determining the degree of sugar processing.

The results show that folding of individual glycopolypeptides begins on the nascent chain and continues for several min post-translationally. Full length intermediates with different disulfides bonding patterns can be distinguished, as well as monomeric fully oxidized subunits prior to homo-oligomer formation. While the folding process takes place in the ER, the assembly of mature oligomers seems to occur mainly in the intermediate compartment between the ER and the Golgi complex.

The rate of folding and its efficiency depends on the conditions in the ER. These vary between cell types and physiological state. The important variables include the redox state(folding can be reversibly inhibited by addition of membrane permeable reducing or oxidizing agents such as DTT or diamide), the presence of lumenal ATP, the concentration of Ca^{++}, the level of ER-chaperons and folding enzymes such as BiP/GRP78 and protein disulfide isomerase, and temperature(folding is more efficient the lower the temperature). Frequently conditions arise in which a fraction of a given protein synthesized misfold while the rest fold properly, emphasizing the stochastic nature of the maturation process. Our results, moreover, demonstrate that the ER is a highly dynamic folding compartment where folding, unfolding, refolding, misfolding. oligomerization, aggregation and disassembly are continuously occurring catalyzed by a variety of folding factors and chaperons.

The quality control system(s), responsible for the retention and retrieval of defective proteins operates at different levels of the pathway: the rough ER, the intermediate compartment and the cis-Golgi. These proteins usually associate with BiP/GRP78. At a slow rate, some move from the ER to the Golgi complex from which they are recycled back to the ER. BiP/GRP78 is likely to play a central role in many aspects of quality control in addition to its role in translocation and folding.

Protein: Protein Interactions in Targeting and Transport: Heat Shock Proteins and Molecular Chaperones

H 002 THE PATHWAY OF CHAPERONE-ASSISTED PROTEIN FOLDING, F.U. Hartl, Rockefeller Research Laboratories, Sloan-Kettering Institute, 1275 York Avenue, New York, N.Y. 10021

Protein folding within cells appears to be mediated by so-called molecular chaperones, mostly constitutively-expressed stress proteins which occur in the cytosol as well as within subcellular compartments. We are interested in understanding the function of members of the hsp70 and hsp60 families of heatshock proteins in de novo protein folding as well as their role in maintaining the functional integrity of preexisting proteins under stress conditions.

Recent evidence suggests that hsp70 and hsp60 components act sequentially in protein folding determined by their differential specificity for structural elements exposed at different stages of the folding pathway. Hsp70 recognizes completely unfolded polypeptide chains as they emerge from ribosomes or at the trans-side of membranes following translocation into organelles such as mitochondria. The newly-made proteins, adopting the conformation of partially folded intermediates, are then transfered to hsp60 which is required for their folding and assembly to the native state. Proteins appear to acquire stable tertiary structure by an ATP-dependent folding process within the central cavity of the hsp60 cylinder (1, 2). The eukaryotic cytosol contains a TCP-1 related double-ring complex (TRiC) which may have hsp60-like function in this compartment (3).

Using the isolated heatshock proteins of E. coli we have reconstituted hsp70/hsp60 mediated protein folding reactions in vitro (4). These experiments show that hsp70 (the DnaK protein of E. coli) functionally depends on two additional heatshock proteins, DnaJ and GrpE. DnaJ and DnaK cooperate in stabilizing the folding polypeptide as a compact intermediate. GrpE, in the presence of DnaJ, stimulates the ATPase activity of DnaK which allows the transfer of the associated substrate to hsp60 (the groEL protein of E. coli). This hierarchical action of chaperone components may represent a major route for the folding of newly-synthesized proteins.

For the hsp60 of mitochondria we have recently demonstrated an additional function in preventing the denaturation of mitochondrial proteins under heat stress (5). Hsp60 binds the unfolded or partially unfolded chains of thermolabile proteins, thus preventing their aggregation, and mediates their refolding at elevated temperature. Hsp60 is unable to interact with these proteins once misfolding and aggregation have occurred.

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H 003 THE ROLE OF hsp90 IN FUNCTION AND TRANSPORT OF THE GLUCOCORTICOID RECEPTOR AND pp60src, William B. Pratt, Kevin A. Hutchison, Louis P. Stancato, Yu-Hua Chow and Richard Jove, The University of Michigan Medical School, Ann Arbor, MI 48109-0626.

Steroid receptors are recovered from hormone-free cells in a heterocomplex containing hsp90 and hsp56, the latter being an immunophilin of the FK506 binding class. Heterocomplexes isolated from cells where the unliganded receptor is nuclear contain substantial amounts of hsp70, as well. It has been shown that a hetercomplex containing hsp90, hsp70, hsp56 and a non-heat shock protein p50 exists in cytosols independent of steroid receptors. The glucocorticoid receptor (GR) is bound to the heat shock complex via an association of the hormone binding domain (HBD) with hsp90, and binding of steroid promotes a temperature-dependent dissociation of the receptor from hsp90. The oncogenic tyrosine kinase $pp60^{src}$ is also bound tightly to hsp90 but in a heterocomplex that contains p50. It is thought that the steroid receptors and several viral protein tyrosine kinases are transported in the cell in the heterocomplex form, and we have proposed that the heat shock protein complex may act as a *transportosome* or general transport particle for a wide variety of proteins as they undergo trafficking through the cytoplasm.

We have shown that hormone-free glucocorticoid receptors that are immunoadsorbed to protein-A-Sepharase or bound to DNA-cellulose become associated with hsp90 when they are incubated with reticulocyte lysate. This is a temperature-dependent and ATP-dependent process that requires monovalent cation (K^+ and NH_4^+ are active, Li^+ and Na^+ are inactive) with the HBD alone being sufficient for association with hsp90. In this cell-free system, the GR becomes associated with a multiprotein complex containing hsp90, hsp70, and hsp56. At this time, we favor a model of heterocomplex assembly in which the HBD of the GR is unfolded and becomes attached to a preformed heat shock protein heterocomplex rather than an ordered assembly of individual proteins into a heterocomplex unit. Reconstitution of the GR-hsp90 complex is accompanied by inactivation of receptor DNA-binding activity and conversion of the receptor from a non-steroid-binding to a high affinity steroid-binding conformation. Thus, the receptor is restored to a functional state characteristic of the untransormed native heterocomplex.

Recently, we have used this cell-free system to reconstitute the multiprotein complex between $pp60^{src}$, hsp90 and p50. This is also an enzymatic, ATP-dependent process that is cation-selective. Because the complex that is created in reticulocyte lysate is very stable, we are using this sytem to see if we can form complexes with proteins that may enter into less stable native heterocomplex forms that do not survive our usual detection procedures. An example is presented by fusion proteins containing the HBD of the estogen receptor (ER) where the native heterocomplexes cannot be detected despite their predicted existence in the intact cell. In the cell-free system, we have formed heterocomplexes with a ß-galatosidase-ER.HBD fusion protein. Based on this observation, we have also assembled cRaf-1 (a protein that is not known to be bound to hsp90 in the cell) into a heterocomplex with hsp90, hsp70 and p50. We feel that, taken together, these observations support a general role for the heat shock protein complex in protein transport. (Supported by NIH grants CA28010 and DK31573 to W.B.P., and CA47809 to R.J.)

Mechanisms of Protein Targeting and Membrane Traversal

H 004 THE NUCLEAR PORE: STRUCTURE, FUNCTION, AND ASSEMBLY, Douglass J. Forbes, Colin Macaulay, Eva Meier, Maureen Powers, and Tam Dang, Department of Biology, University of California at San Diego, La Jolla, CA 92093.

Nuclear pore proteins bearing N-acetylglucosamine residues have been shown to be essential for nuclear transport; the lectin WGA binds to these proteins and blocks transport. Xenopus eggs contain 3 major WGA-binding proteins (WGAbps) and several minor ones. Antibodies were obtained to the major WGAbps, p200, p97, and p60. Immunofluorescence with the individual antisera on nuclei reconstituted in vitro each gave a punctate nuclear rim staining, characteristic of nuclear pores. To date, immunoEM confirms p200 and p60 to be pore proteins. To address the assembly and disassembly of the nuclear pore, we asked whether these pore proteins are modified in a cell cycle-dependent manner, focusing first on potential phosphorylation. We found that in a Xenopus egg extract which cycles repeatedly between interphase and mitosis, p200 and p97 corresponds exactly to the peak of mitotic activity of MPF, the cdc2/cyclin kinase of Xenopus. Phosphorylation of p200 and p97 also can be induced in interphase extracts of Xenopus eggs when cyclin is added (cyclin combines with endogenous cdc2 to form MPF). The cell-cycle dependent modification of nuclear pore proteins suggests that phosphorylation may induce disassembly of the pores at mitosis. To determine whether mitotic phosphorylation prevents the assembly of these proteins into pores, we first assembled "pore-free" nuclei (Newport and Dunphy, J.C.B., 1992). Sperm chromatin was decondensed in a crude preparation of nucleoplasmin, followed by membrane vesicle addition. The vesicles bound to chromatin and, in the presence of ATP and GTP, fused to form a double nuclear membrane. When such pore-free nuclei were isolated and added to cytosol containing pore glycoproteins, the nuclei acquired a punctate nuclear rim stain upon immunofluorescence with anti-pore antisera. If, however, the cytosol contained phosphorylated pore proteins, no rim stain was observed. These data argue that pore formation involves an assembly step inhibited by phosphorylation. Implications for the mechanism of assembly of the nuclear pore will be discussed.

H 005 PROTEIN TRANSLOCATION INTO THE ENDOPLASMIC RETICULUM, Peter Mayinger, Adam J. Savitz and David I. Meyer, Department of Biological Chemistry, UCLA School of Medicine, and the Molecular Biology Institute, Los Angeles, CA, 90024

Translocation of nascent secretory and membrane proteins across the membrane of the rough endoplasmic reticulum (ER) represents the first step in protein transport through the secretory pathway. Signal-sequence mediated recognition and targeting of nascent polypeptides to the ER membrane is catalyzed by a series of receptors, while the actual translocation appears to make use of a pore or channel in the lipid bilayer. Stress-protein catalyzed folding of the nascent chain most likely provides the driving force for the vectorial transfer of the polypeptide through the pore and into the lumen of the rough ER.

We have characterized two activities that are required for the translocation reaction. One is a 180 kDa integral membrane protein that is involved in the binding of ribosomes to mammalian ER. Recent data indicate that this ribosome receptor (p180) is essential for translocation. Monoclonal anti-p180 antibodies inhibit both ribosome binding and translocation in intact microsomes, while immuno-depletion of p180 from extracts used to prepare translocation-competent proteoliposomes results in depletion of both ribosome binding and translocation activity. The other activity is an ATP transporter in yeast microsomes, whose inhibition results in a loss of translocation competence. This finding is consistent with the postulated role for luminal ATP-requiring stress proteins (such as Kar2p/BiP) in the translocation process.

H 006 ENZYME MECHANISM OF *E. COLI* PREPROTEIN TRANSLOCASE, Bill Wickner, Molecular Biology Institute, University of California, Los Angeles.

Preproteins are stabilized by chaperones such as SecB, then bind to translocase, a multisubunit membrane protein. The SecA subunit of translocase recognizes both the leader and mature domains of the preprotein as well as SecB. SecA then couples the energy of ATP binding to initiating translocation. The translocation process drives the unfolding of the preprotein tertiary structure. Translocation is driven by two energy sources, ATP and the membrane protonmotive force. Photoactivatable crosslinkers, attached at specific points to translocation-arrested preproteins, have been used to map the pathway by which a preprotein crosses the membrane. We find that this path lies entirely within the SecA and SecY subunits of translocase, shielded from the other subunits of translocase and from the membrane lipid phase.

Protein Degradation in the Cytosol and Organelles (Joint)

H 007 SELECTIVE DEGRADATION OF CYTOSOLIC PROTEINS BY LYSOSOMES, J. Fred Dice, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111

Lysosomes are able to take up and degrade cytosolic proteins by several different pathways. Although lysosomal pathways of protein degradation have traditionally been viewed as degrading proteins to their constituent amino acids, it is now clear that certain peptides are spared degradation, released from the lysosomal compartment, and eventually released from cells. Some of these peptides are of an appropriate size to serve as antigens. We have studied a lysosomal pathway of proteolysis that is selective for cytosolic proteins containing peptide sequences biochemically related to Lys-Phe-Glu-Arg-Gln (KFERQ). This pathway is stimulated in confluent cell cultures that are deprived of serum growth factors and in liver and certain other tissues of fasted animals. Isolated lysosomes are able to selectively take up and degrade KFERQ-containing proteins, and this process is stimulated by ATP/$MgCl_2$ and the constitutively-expressed heat shock 70 kD protein (hsc73). Other members of the heat shock 70 protein family do not bind to KFERQ-containing proteins and do not stimulate uptake and degradation of proteins by isolated lysosomes. This lysosomal pathway of proteolysis is saturable and temperature-dependent. Intralysosomal degradation of the protein, but not lysosomal uptake of the protein, is inhibited by ammonium chloride and leupeptin. At 0° C. proteins with KFERQ-like peptide sequences specifically bind to a protein on the lysosome surface, and such binding is stimulated in the presence of hsc73. This lysosomal binding protein is probably a receptor or a component of the protein translocation machinery within the lysosomal membrane. A heat shock 70 kD protein also resides within the lysosome, and this protein may play a role in the import or the intralysosomal digestion of substrate proteins.

Mediation and Regulation of Intracellular Membrane Traffic

H 008 G PROTEIN REGULATION OF VESICULAR TRANSPORT BETWEEN THE ENDOPLASMIC RETICULUM (ER) AND GOLGI COMPARTMENTS. [1]William E. Balch, [1]Helen Plutner, [1]Ruth Schwaninger, [1]Frank Peter, [1]Ellen J. Tisdale, [1]Jeffrey Bourne, [2]Michael McCaffery, [2]Marilyn G. Farquhar, [1]Howard W. Davidson. [1]The Scripps Research Institute, La Jolla, CA 92037; [2]University of California San Diego, La Jolla, CA 92093.

Vesicular transport of protein between exocytic compartments of eukaryotic cells requires the function of GTP-binding proteins. These proteins are likely to participate at different steps involved in the cyclical formation, targeting and fusion of carrier vesicles. Using model systems in which the function of GTP-binding proteins in the vectorial transport of protein between the ER and multiple Golgi compartments can be reconstituted in vitro, we can now define the regulation of transport by GTP-binding proteins at the different steps involved in vesicle budding, targeting and fusion. Export from the ER was sensitive to reagents which perturb the function of heterotrimeric G proteins and the small GTP-binding protein ARF (ADP-ribosylation factor). Similarly, an antibody which neutralizes the function of the ras-related small GTP-binding protein rab1b prevented exit of VSV-G protein from the ER. In contrast, VSV-G protein was exported from the ER to punctate pre-Golgi intermediates in the presence of mutant rab1 proteins defective in GTP-binding and hydrolysis. The site of accumulation was similar to that blocked by a peptide reagent which antagonizes the interaction of rab1 with downstream effectors and by an antibody specific for the late-acting transport component NSF (SEC18). Current evidence suggests that rab1 functions in the context of biochemical cycle in which recruitment and function are temporally distinct: the role of rab1 may be to regulate the cyclical assembly, targeting and/or disassembly of coat complexes involved in vesicle formation. These results provide evidence that multiple small GTP-binding proteins (rab1a, rab1b, ARF, rab2, (SAR1)) regulate the maturation of protein from the ER through early compartments of the secretory pathway. They in addition suggest a model in which export from the ER is "gated" through the active participation of heterotrimeric G proteins.

H 009 BIOGENESIS OF NEUROSECRETORY VESICLES, Wieland B. Huttner, Institute for Neurobiology, University of Heidelberg, Im Neuenheimer Feld 364, D-6900 Heidelberg, Germany.

The following aspects concerning the biogenesis of neurosecretory vesicles will be discussed:

1. Secretory Granules / Large Dense Core Vesicles:
   - 1.1 Milieu-induced aggregation of regulated secretory proteins in the trans-Golgi network (1).
   - 1.2 Membrane association of regulated secretory proteins in the trans-Golgi network (2).
   - 1.3 Role of multiple heterotrimeric G-proteins and other GTP-binding proteins in the formation of secretory vesicles from the trans-Golgi network (3, 4).
   - 1.4 Inhibition of formation of secretory vesicles from the trans-Golgi network by brefeldin A (5).
2. Small Synaptic Vesicles / Synaptic-like Microvesicles
   - 2.1 Membrane traffic route to synaptic-like microvesicles (6).
   - 2.3 Characterization of synaptic-like microvesicles as neurotransmitter storage organelles in comparison with secretory granules.

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H 010 MECHANISM AND REGULATION OF VESICLE BUDDING, Randy Schekman, Charles Barlowe, Rainer Duden, Midori Hosobuchi, Nancy Pryer, Michael Rexach, Nina Salama, and Thomas Yeung, Department of Molecular and Cell Biology, Howard Hughes Medical Institute, Barker Hall, University of California, Berkeley, CA 94720.

A set of at least seven genes (SEC12, SEC13, SEC16, SEC23, SEC24, SEC25, SAR1) is required for transport vesicle budding from the endoplasmic reticulum in *S. cerevisiae*. This process has been reproduced with isolated ER membranes and pure cytosolic and peripheral membrane Sec proteins. The budding reaction is monitored by the transfer of a radioactive secretory protein, yeast α-factor precursor, from rapidly-sedimenting ER membranes to slowly-sedimenting transport vesicles. Isolated transport vesicles are devoid of three different Sec proteins that are required for budding (Sec12p, Sar1p, Sec13p), but contain Sec proteins that are required for vesicle targeting (Sec22p, Ypt1p). Starting with urea- and salt-washed membranes, budding requires three soluble protein fractions (Sar1p, Sec13/25p complex, and Sec23/24p complex) and hydrolyzable ATP and GTP. An integral membrane protein, Sec12p, contains an N-terminal, cytosolically-exposed domain that is also required for budding. Deletion of the membrane anchor and C-terminal lumenal domain of Sec12p generates a soluble N-terminal fragment that is a potent inhibitor of budding. The target of this inhibitory effect is Sar1p; addition of excess Sar1p overcomes inhibition. Sar1p, Sec12p, and Sec23p are linked in a cycle of GTP hydrolysis that is coupled to the budding event. Sar1p performs slow GTP hydrolysis and nucleotide exchange that require detergent or phospholipid. GTP hydrolysis by Sar1p is stimulated ten to fifteen fold by the Sec23p subunit of the 23/24p complex. GTP-GDP nucleotide exchange on Sar1p is stimulated five fold by the cytosolic domain of Sec12p. The signal that triggers nucleotide exchange, the target of GTP-Sar1p, and the mechanistic coupling to vesicle budding are open questions.

SEC21, another gene implicated in protein transport from the ER, encodes a 105 kD polypeptide that is a subunit of a yeast homolog of the non-clathrin coat protein complex (coatomer). Among the other subunits of the yeast complex (α, 150 kD; β, 110 kD; γ, Sec21p; δ, 73 kD; ε, 35 kD, and μ, 25 kD), the β subunit has been cloned and shown to be ~40% identical in amino acid sequence to the corresponding β-COP from mammals. Coatomer is resolved from the three protein fractions required for vesicle budding, however a fraction of the Sec21p persists in the urea- and salt-washed membranes used to assay vesicle formation. Alternative methods will be necessary to examine the role of the coatomer in the budding reaction.

Mechanisms of Endocytosis and Membrane Recycling (Joint)

H 011 REGULATION OF ENDOCYTIC FUSION EVENTS IN VITRO, Jean Gruenberg. European Molecular Biology Laboratory. Postfach 10.2209, D-6900 Heidelberg. Germany.

We are studying the mechanisms of membrane-membrane interactions in the endocytic pathway with an in vitro fusion assay. Until now, we have reconstituted two separate endosomal fusion events, which reflect in vivo an early and a late stage of the pathway, respectively. The first one corresponds to the lateral (homotypic) fusion of early endosomes with each other (an observation suggesting that early endosomes form a highly dynamic network in vivo). We have shown that this process is specific, microtubule-independent, and regulated by the small GTP-binding protein rab5. In addition, this fusion event is inhibited by mitotic cytosol, a finding consistent with membrane trafic arrest during mitosis in mammalian cells. This inhibition is mediated by the cdc2 kinase specifically associated to cyclin B, but not cyclin A. The second fusion event occurs during transport from peripheral early endosomes to perinuclear late endosomes. We previously observed that microtubule-dependent endosomes with a multivesicular appearance are intermediates in this transport. We refer to them as endosomal carrier vesicles (ECVs). In vitro, we have reconstituted the fusion of ECVs with late endosomes both in polarized epithelial cells and in non polarized cells. This process is specific, facilitated by the presence of polymerized microtubules, and depends on MAPs and motor proteins.
Until now, studies in membrane transport have revealed the existence of complex mechanisms for the regulation of membrane-membrane interactions. However, relatively little is known about these interactions. We have, therefore, used an assay that measures the transfer of membrane-associated proteins from "donor" to "acceptor" endosomes upon fusion. [This assay fulfills all criteria of the fusion process.] We observed that transfer/fusion still occurred when donor endosomes were sonicated into small (0.1μm diameter) vesicles before the assay. Then, only 5 proteins were efficiently transferred, including two membrane proteins and three peripheral proteins associated to the membrane cytoplasmic face. Partial sequencing showed one of the latter protein to be annexin II, which has been previously implicated in membrane-membrane interactions. Our data, suggest that annexin II, together with the 4 other proteins we have identified, may be involved in the formation of close contacts between membranes and/or fusion.

H 012 MEMBRANE TRAFFIC AND TRANSCYTOSIS IN POLARIZED EPITHELIAL CELLS: SIGNALS, MECHANISMS, AND REGULATION. K. Mostov, G. Apodaca, B. Aroeti, C. Okamoto, W. Song, and *M. Bomsel, Departments of Anatomy, and Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0452, USA, and *Unité de Pathogénic Microbienne Moléculaire, Institut Pasteur, Paris, France.

Polarized epithelial cells use two mechanisms to send proteins to the correct plasma membrane domain: direct delivery from the TGN to the final surface, and indirect delivery to one surface, followed by endocytosis and transcytosis to the opposite surface. We have used the polymeric immunoglobulin receptor (pIgR) as a model to study these processes. The membrane-proximal 17 residues of the cytoplasmic domain of the pIgR are an autonomous signal for delivery from the TGN to the basolateral surface. Transcytosis is regulated by two independent signals: phosphorylation of Ser^{664} on the cytoplasmic domain, and binding of ligand (IgA) to the extracellular domain. Activation of the heterotrimeric G protein, G_s, by cholera toxin stimulates transcytosis independently of these two signals. We have reconstituted budding of transcytotic vesicles from early endosomes in a perforated cell system. Both the α and βγ subunits of G_s act to control sorting of pIgR into transcytotic vesicles. G_s is thus the first identified component of the machinery for polarized sorting of proteins in epithelial cells

Molecular Motors, Cables, Tubules and Filaments in Membrane Traffic

H 013 THE ACTIN CYTOSKELETON OF BUDDING YEAST AND ITS ROLE IN MORPHOGENESIS,

David Drubin, Doug Holtzman, Tom Lila, Anne Moon, Dani Vinh, Matt Welch, Shirley Yang, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

In budding yeast, asymmetric localization (or activation) of cell surface proteins, including enzymes involved in cell wall remodeling and (in mating cells) pheromone receptors and adhesion molecules, is required for polarized growth and mating. The actin cytoskeleton plays a central role in the develpment of these asymmetries; actin and actin-binding protein mutants are defective in development of surface polarity. We have used actin filament affinity chromatography and biochemical assays to identify yeast actin-binding proteins. Yeast actin and the actin-binding proteins cofilin, fimbrin, and Abp1p have all been shown to be concentrated at the growing region of the cell cortex. Genetic studies support the roles of these proteins in polarized growth.

The *ABP1* gene encodes a 65 kD protein. When overproduced, Abp1p causes actin to inappropriately assemble on surfaces of the mother cell rather than exclusively in the bud as is normal for wild type cells. This causes cell surface growth, normally restricted to the bud, to occur in the mother cell. Deletion of the *ABP1* gene was found to have no effect on the polarized growth of the yeast cell. However, a genetic screen to identify mutations that enhance the severity of a deficiency in Abp1p identified two *SLA* genes. *sla* mutants are defective in assembly of the cortical cytoskeleton. Moreover, at the non-permissive temperature *sla* mutants grow isotropically rather than in a polarized manner.

H 014 *IN VIVO* AND *IN VITRO* STUDIES ON THE STRUCTURE AND FUNCTION OF MYOSIN,

Thomas T. Egelhoff, Randall J. Lee, Bruce Patterson, Kathleen M. Ruppel, Taro Q.P. Uyeda, and James A. Spudich, Departments of Biochemistry and Developmental Biology, Stanford Univer School of Medicine, Stanford, CA 94305.

Myosin is a ubiquitous eukaryotic molecular motor that moves actin filaments in an ATP-dependent manner. Multiple approaches have been used to implicate myosin in a variety of nonmuscle movements such as cytokinesis, cell migration, capping of cell surface receptors, intracellular vesicle movement, and developmentally-associated morphogenetic shape changes. We are using the simple eukaryote *Dictyostelium discoideum* to further explore the role that myosin plays in these motile behaviors. *Dictyostelium* cells lacking the endogenous myosin heavy chain gene (created by gene replacement) exhibit striking defects in several types of cellular motility. This mutant background is used as a recipient for altered myosin heavy chain genes, and the effects of each introduced change on *in vivo* and *in vitro* myosin function are assayed. Various mutagenesis techniques have been employed to alter either the head of myosin (Subfragment-1, or S-1),which is the motor unit of the molecule, or the alpha-helical coiled-coil tail, which governs assembly of myosin into thick filaments.

Analysis of the tail domain of myosin has included serial truncations to determine regions of the tail necessary for filament assembly, as well as site-directed mutagenesis of phosphorylatable residues that have been implicated in control of assembly. Truncation studies have mapped the region of the tail necessary to drive filament formation *in vitro* to within 33 amino acids. Myosins bearing tails which are truncated N-terminal to this region are not competent to assemble, and are not able to complement the myosin-specific defects of the null strain, whereas myosins with tails truncated C-terminal to this domain are able to form filaments *in vivo* and *in vitro*. Site-directed mutagenesis studies have explored the consequences of converting phosphorylatable threonine residues either to alanine residues, thereby eliminating phosphorylation at these positions, or to aspartate residues, which mimics the negative charge state of the phosphorylated molecule at these positions. Replacement of the phosphorylatable threonines with alanines does not effect the *in vitro* assembly of the myosin, but results in substantial overassembly of the myosin into the cytoskeleton *in vivo*. Cells are still able to use this myosin to drive capping of cell surface receptors, cytokinesis, and morphological changes during development. Replacement of the phosphorylatable threonines with aspartate eliminates filament assembly *in vitro* and renders the myosin unable to drive any tested contractile events *in vivo*. These results demonstrate that heavy chain phosphorylation plays a key modulatory role in controlling the recruitment and assembly of myosin within the cell.

Site-directed mutagenesis of the motor domain initially explored regions thought to be important for ATP and actin interactions. This approach yielded mutated myosins that are no longer able to bind nucleotide but which bind actin tightly (rigor binders), myosins that have a decresed affinity for nucleotide or decreased ATP turnover, and myosins with decreased stability *in vivo* and *in vitro*. *In vivo* effects of these various mutations ranged from slight temperature sensitivity to a fully null phenotype, depending upon the severity of the biochemical defect. Recent efforts have involved creating banks of random point mutations over short stretches of highly conserved myosin sequence throughout the myosin head. Three general phenotypic classes have emerged from this approach: wild-type like cells (normal cytokinesis and development *in vivo*) that express myosins with no or only minor biochemical defects; intermediate cells (defective or unreliable cytokinesis and development *in vivo*) that express myosins with a variety of biochemical defects, including lowered actin-activation of ATP turnover and uncoupling of ATP hydrolysis and motility *in vitro*; and null-like cells (no cytokinesis and early developmental arrest *in vivo*) that express myosins that are specifically unable to hydrolyze ATP. In collaboration with Dr. Ivan Rayment (U. Wisconsin, Madison), we are currently trying to place each of these changes on the high resolution three-dimensional structure of S1 (recently determined by Dr. Rayment and his colleagues). We are also collaborating with Dr. Ken Holmes' group (Max-Planck-Institute, Heidelberg) and Dr. Dietmar Manstein (NIMR, Mill Hill, London) to analyze the position of these mutations in relation to the docked structure of S1 bound to actin in the actin-S1 complex formed in the absence of ATP. These studies should provide a framework for understanding how myosin links ATP hydrolysis to movement and force production, and how this motor function leads to motile activities *in vivo*.

Workshop I: GTP Binding Proteins in Vesicular Traffic

H 015 RAB9 FUNCTIONS IN TRANSPORT BETWEEN LATE ENDOSOMES AND THE TRANS GOLGI NETWORK,

Thierry Soldati, Markus A. Riederer, Joseph Lin, Allan Shapiro and Suzanne R. Pfeffer. Department of Biochemistry, Beckman Center B400, Stanford University School of Medicine, Stanford, CA 94305-5307

Mannose-6-phosphate receptors (MPRs) cycle between the trans Golgi network (TGN) and late endosomes to deliver newly synthesized, soluble lysosomal enzymes to pre-lysosomes. We have established a cell free system that reconstitutes the transport of MPRs between late endosomes and the TGN. Small, ras-like, GTP binding proteins have been demonstrated to be involved in a growing number of vesicle trafficking pathways. In collaboration with Marino Zerial and Daniela Lombardi (EMBL, Heidelberg), we investigated the potential role of rab7 and rab9 proteins in endosome→TGN transport (1). Lombardi and Zerial have shown that rab7 and rab9 are present on late endosomes; rab9 is also on the TGN. Results from two types of *in vitro* experiments suggest that rab9 plays a role in MPR recycling. First, anti-rab9 antibodies inhibit transport up to ~50% in a concentration-dependent manner, under conditions in which anti-rab7 antibodies have no effect. Second, cytosols containing overexpressed rab9 protein, or supplemented with purified rab9 protein, had a two-to-three-fold higher specific activity than either control cytosol, or cytosol containing overexpressed rab7 protein. These experiments show that rab9 is a limiting component in CHO cytosol that can facilitate endosome→TGN transport.

The in vitro activity of rab9 requires an intact carboxy terminus and prenylation by geranylgeranyl transferase present in cell extracts. Gel filtration chromatography revealed that preincubation of rab9 protein, purified from an E. coli-overexpressing strain, with cytosol, ATP, and geranylgeranyl diphosphate, led to its assembly into a cytosolic complex with an apparent molecular mass of ~80kD. Complex formation required the presence of an intact carboxy terminus, geranylgeranyl diphosphate, cytosol, and incubation at 37°C. The bulk of the low molecular weight cytosolic GTP binding proteins displayed similar chromatographic properties as the ~80kD rab9 complexes. These findings suggest that like rab3a and Sec4p, rab9 interacts with GDI to maintain its solubility and facilitate its recycling between donor and acceptor membranes. Indeed, purified, prenylated rab9 protein could be shown to form an ~80kD complex with rab3a GDI purified from bovine brain. Experiments are in progress to test our working hypothesis that rab9 functions in the process by which transport vesicles recognize the TGN as the correct fusion target.

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H 016 REGULATION OF MEMBRANE TRAFFIC BY SMALL GTP-ASES IN EPITHELIAL CELLS, Anne Lütcke[1], Sanna Janssen[2], Robert G. Parton[1], Cecilia Bucci[1], Lukas Huber[1], Eero Lehtonen[2] and Marino Zerial[1], [1]European Molecular Biology Laboratory, D-6900 Heidelberg, Germany, [2]University of Helsinki, SF-00290 Helsinki 29, Finland.

The rab subfamily of small GTPases has been implicated in the regulation of membrane traffic in eukaryotic cells. About 30 members of this subfamily have been identified and some of them have been localized to distinct intracellular membrane compartments. These proteins have been proposed to confer specificity and unidirectionality on vesicular transport between the organelles harbouring them. We investigated the intracellular localization and function of rab5. This protein is found associated with the plasma membrane, early endosomes and clathrin-coated vesicles. Expression of the GTP-binding defective mutant rab5Ile133 in BHK cells led to a 50% decrease in the rates of uptake of transferrin and of a fluid phase endocytic marker, horseradish peroxidase (HRP), compared to control cells. Conversely, overexpression of wt rab5 was able to increase these rates. The morphology of early endosomes was also drastically affected. The mutant proteins induced fragmentation of early endosomes while overexpression of wild type rab5 led to the appearance of atypically large early endosomes.

These data suggest that rab5 is a rate-limiting regulatory protein which controls the dynamics of early endosome fusion and the fusion of plasma membrane derived endocytic vesicles with early endosomes. We are currently investigating the functional mechanism of rab5 by identifying its interacting components.

We then studied the involvment of rab proteins in the regulation of membrane traffic in epithelial cells. Polarized epithelial cells are organized into apical and basolateral surface domains facing two different extracellular environments.The differentiation into apical and basolateral transport pathways requires the presence of additional components of the trafficking machinery with respect to non-polarized cells. We addressed this problem by searching for rab proteins which might be specifically expressed in epithelial cells. Using a PCR approach we identified 12 novel rab proteins from mouse kidney. Northern blot analysis was employed to investigate the expression of these proteins in various mouse organs. The transcript of rab17 was detected exclusively in kidney, liver and intestine, which are tissues enriched in epithelial cells. To establish whether rab17 is specifically expressed in epithelial cells we studied its expression in the developing kidney. Rab17 was not expressed in the mesenchymal non-epithelial precursors but was detected after differentiation into epithelial cells, at the onset of the polarization process. *In situ* hybridization studies showed that rab17 is restricted to epithelial cells in the embryonic kidney and intestine. Using specific affinity-purified antibodies in immunofluorescence microscopy studies, rab17 was found expressed at high level in the epithelial cells of the proximal tubules, at lower level in distal tubules and absent from glomeruli and endothelial cells. Electron microscopy analysis indicated that rab17 is located on the basolateral plasma membrane and on tubulo-vesicular structures underlying the apical brush border. These results indicate that rab17 is a novel GTP-binding protein, specifically expressed in epithelial cells, which could control a transport step necessary for establishment and maintenance of cell polarity.

Workshop II: Involvement of Clathrin in Membrane Traffic

H 017 DIVERSITY AND TARGETING OF COATED VESICLE ADAPTORS. Margaret S. Robinson, Catriona L. Ball, and Matthew N.J. Seaman, University of Cambridge, Cambridge, UK.

Adaptors are the components of clathrin-coated vesicles that attach the clathrin outer cage to the vesicle membrane. There are two types of adaptors in the cell, one associated with the plasma membrane and another associated with the trans-Golgi network (TGN). The plasma membrane adaptor contains an α-adaptin subunit and a β-adaptin subunit, while the TGN adaptor contains a γ-adaptin subunit and a β'-adaptin subunit. All three types of adaptins, α, β, and γ, have a similar three domain structure, consisting of N terminus, hinge, and C-terminal "ear". There are two α-adaptin genes which appear to be co-expressed in most tissues, but one of the genes undergoes alternative splicing in brain. Antibodies have been raised against the various α-adaptin isoforms, using fusion proteins as antigens, to compare their distribution in cells and tissues. Southern blotting suggests that there may also be a second γ-adaptin gene. To look for targeting signals on the plasma membrane and TGN adaptor complexes, the ears and/or hinges were swapped between α- and γ-adaptins and the constructs localised with a species-specific antibody against the γ-adaptin hinge. These studies reveal that the α- and γ-adaptin ears are not required for targeting and indicate that the signal is likely to be in the adaptor "head". Adaptor targeting has been reconstituted in vitro, using permeabilised acceptor cells and donor cytosol, and labelling with species-specific or tissue-specific antibodies that only recognise donor α- or γ-adaptins. Recruitment of both plasma membrane and TGN adaptors onto the appropriate membrane is enhanced by the addition of energy, and TGN adaptor labelling is increased still further by the addition of GTPγS. A more quantitative binding assay has been developed for the TGN adaptors, using radioiodinated purified proteins. Recruitment was found to be increased about three-fold by the addition of cytosol. We now plan to use this system to look for the cytosolic and membrane proteins involved in TGN adaptor recruitment. GTPγS has a completely different effect on the recruitment of plasma membrane adaptors: it appears to block the binding of adaptors to the plasma membrane and causes them to go to a perinuclear endosomal compartment instead. These results suggest that GTPγS may act as a switch, activating some adaptor receptors and inactivating others.

H 018 **GTP-BINDING PROTEINS PARTICIPATE AT DISTINCT STAGES IN CLATHRIN COATED VESICLE-MEDIATED ENDOCYTOSIS,** Thomas E. Redelmeier, Laura L. Carter, Elizabeth Smythe, Christophe Lamaze, Takeshi Baba, Leslie A. Woollenweber, Ellen J. Tisdale, Alex M. van der Bliek* and Sandra L. Schmid. Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, 92037 and *Division of Biology, California Institute of Technology, Pasadena, CA.

Our laboratory has established and extensively characterized a novel cell-free assay system using perforated human A431 cells which faithfully reconstitutes receptor-mediated endocytosis of transferrin (Tfn) (1,2) and more recently, epidermal growth factor (EGF). To complement this *in vitro* system for endocytosis, we have also developed stage-specific assays which enable detection of three biochemically distinct events involved in coated vesicle formation: coat assembly, coated pit invagination and coated vesicle budding. The following biochemical and morphological properties have established the physiological validity of this model system and these biochemical assays for receptor-mediated endocytosis. 1) Extensive morphological characterization using both HRP and gold-labelled ligands has shown that endocytosis occurs via clathrin coated pits. 2) Coated pit assembly, invagination and coated vesicle budding require elevated temperatures and ATP hydrolysis. 3) Endocytosis *in vitro* is inhibited by anti-clathrin antibodies and by antibodies directed against the cytoplasmic domain of the transferrin-receptor (Tfn-R). 4) Purified AP2 adaptors (plasma membrane-specific coat constituents) stimulate Tfn-sequestration and this stimulation requires cytosolic clathrin. 5) Other, as yet unidentified cytosolic factors are required at multiple stages in coated vesicle formation. 6) Multiple GTP-binding proteins participate at distinct stages in coated vesicle formation. Here we report that one of these GTP-binding proteins is dynamin, originally identified as a microtubule-associated mechano-chemical motor protein (3), but more recently implicated as having a role in endocytosis (4, 5).

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Peptide Translocators and Pore-Forming Molecules (Joint)

H 019 SUBSTRATE SPECIFICITY OF HEMOLYSIN TRANSPORT, Fang Zhang, Jonathan Sheps, David Greig, Ya Yin, Cheryl Arrowsmith and Victor Ling. The Ontario Cancer Institute, Department of Medical Biophysics, University of Toronto, Toronto, Ontario, CANADA.

The secretion of the 107kDa hemolysin A (HlyA) protein from the cytoplasm of E coli directly into the surrounding medium is mediated by membrane proteins hemolysin B (HlyB) and hemolysin D (HlyD). Hemolysin B is a member of the so called ATP-binding cassette (ABC) transporter superfamily which includes the multidrug resistance P-glycoprotein, the cystic fibrosis CFTR protein, and the MHC-associated transporter of antigenic peptides. Recognition of HlyA by the HlyB/D transporter is dependent on a signal sequence mapped approximately to the last 50 amino acids of the C-terminus of HlyA. Many proteins which are not normally secreted by E. coli are secreted by the hemolysin transporter when engineered to contain the C-terminus of HlyA. We show that the recognition of the HlyA signal sequence by the hemolysin transport system may be mediated via specific sites on HlyB. This conclusion is based on experiments in which we show that transport defective mutants of HlyA containing deletions in the signal sequence can be compensated at least in part by reversion point mutations in HlyB. These point mutations all mapped close to the predicted transmembrane domains of HlyB on the cytoplasmic side. In another approach, we show that a 70 amino acid sequence of leukotoxin (LktA) can substitute functionally for the HlyA's signal sequence so that a chimeric molecule of HlyA containing the LktA sequence is transported as efficiently as wild-type hemolysin HlyA. The LktA peptide has no primary sequence similarity to the 50 amino acid signal sequence of HlyA. This finding implies that the transport signal of HlyA is not determined by a unique primary sequence. A secondary structure common to both the HlyA and LktA sequences can be predicted using a computer program involving six different methods. A common structure of helix-turn-helix and strand-loop-strand was predicted. This predicted structure is functionally consistent with many deletion mutants in the signal sequence of HlyA, although it is not consistent with a number of point mutants. Thus we conclude that the HlyA transport signal is complex and may involve an as yet unidentified higher order structure. Since the HlyA and LktA primary sequences are entirely different, it is likely that, despite an apparent similarity in secondary structures, the molecular surfaces presented by these peptides to the transporter will be quite different. This raises the possibility that the hemolysin transporter is able to recognise as substrates an unexpected diversity of structures. Three dimensional structure information at the atomic level is currently being sought for both the HlyA and the LktA signal sequence. An understanding for the basis of the apparent broad specificity of the hemolysin transporter may provide new insights into the mechanism of action of other ABC transporters. For example, it may be speculated that the MHC-linked translocation of a broad range of antigenic peptides from the cytoplasm into the lumen of the endoplasmic reticulum may function in a similar manner. The transporter may recognise antigenic peptides based on some as yet unidentified higher order structures.

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H 020 CFTR MISLOCALIZATION IN CYSTIC FIBROSIS, John R. Riordan, Xiu-Bao Chang, and Norbert Kartner, Research Institute, The Hospital for Sick Children, and the Department of Biochemistry and Clinical Biochemistry, University of Toronto, Toronto, Ontario, Canada.

From the perspective of the topic of this symposium, CFTR is a member of a superfamily of transporters involved in the translocation of a diverse range of substrates including polypeptides across membranes of either intracellular compartments or the cell surface. However, these is as yet no evidence that CFTR transports a polypeptide or any other substrate across the apical membrane of epithelial cells in which it resides. Rather, its primary function seems to be that of a low conductance Cl^- channel subject to a complex mechanism of regulation. In a speculative model, we have proposed earlier that part of this regulatory mechanism may involve an active movement of a unique portion of the protein itself (R domain) against the cytoplasmic aspect of the pore-forming domains. If this were true, CFTR would be at the same time an active transporter and a Cl^- channel. Of greater importance from the perspective of the disease, the mutation in the CFTR gene which occurs in the majority (70%) of patients (ΔF508) severely alters the biosynthesis and intracellular trafficking of the molecule. ΔF508 CFTR is apparently trapped in the ER and degraded; hence, it fails to reach its normal site of action in the apical membrane. We have confirmed that this occurs *in vivo* by directly observing the protein localization using monoclonal antibodies to CFTR. Immunocytochemical analysis of cryosections of skin biopsies from patients homozygous for the ΔF508 mutation showed in epithelial cells of the sweat duct, only weak intracellular staining and a complete absence of the normal strong apical staining. Heterologous expression studies in mammalian and insect cells reveal that this biosynthetic arrest of ΔF508 CFTR occurs in the former but not the latter. Furthermore, when not biosynthetically limited, the mutant protein is functionally competent. This focusses attention on the practical need to understand and maniuplate this biosynthetic block. Experiments in the laboratory of M. J. Welsh at the University of Iowa have already shown that reduced temperature can promote maturation and transport of ΔF508 CFTR to the cell surface. Additional means of accomplishing this are actively being sought. (Supported by the Cystic Fibrosis Foundations of Canada and the U.S. and NIH-NIDDK).

Protein Sorting and Retention in the Exocytic Pathway

H 021 **ESSENTIAL ROLE FOR A PROTEIN KINASE AND A LIPID KINASE COMPLEX IN PROTEIN SORTING**
Scott D. Emr, Jeffrey H. Stack, Kaoru Takegawa and Peter V. Schu, UCSD School of Medicine, La Jolla, CA 92093-0668

Genetic analysis of protein sorting to the yeast lysosome-like vacuole has permitted the isolation of a large number of *vps* (vacuolar protein sorting) mutants which missort and secrete vacuolar hydrolases (1). *vps15* and *vps34* mutants appear to form a unique subset among the *vps* mutant collection. They share a common set of growth, morphology, and protein sorting defects (2,3). The *VPS15* gene encodes a 1,455 amino acid myristoylated phosphoprotein that shares significant sequence similarity (N-terminal 300aa) with the catalytic domains of Ser/Thr protein kinases. The middle third of Vps15p shares homology with the regulatory subunit of protein phosphatase 2A (PP2A). The *VPS34* gene encodes an 875 amino acid protein that is homologous to the catalytic subunit of mammalian phosphatidylinositol 3-kinase (PI 3-kinase) (4). Mammalian PI 3-kinase associates with several cell surface, protein-tyrosine kinase receptors (e.g., the PDGF and CSF-1 receptors). Changes in residues of Vps15p as well as Vps34p that are conserved among protein kinases and lipid kinases, respectively, result in the functional inactivation of the Vps15 and Vps34 proteins (3,4,5). The mutant cells are *ts* for growth and exhibit extreme defects in vacuolar protein sorting.

Genetic and biochemical evidence indicates that the Vps15 protein kinase forms a complex with the Vps34 lipid kinase. Overproduction of Vps34p in *vps15* kinase domain point mutants suppresses the protein sorting defects associated with these mutant strains. A direct association between the Vps15 and Vps34 proteins was demonstrated using both native immunoprecipitation and chemical cross-linking experiments. In addition, both Vps15p and Vps34p cofractionate as membrane-associated proteins (cytoplasmic face of membrane, possibly the Golgi).

Short C-terminal truncations of Vps15p (ΔC30) result in a temperature-conditional defect in vacuolar protein sorting (5). proCPY sorting and maturation are essentially wild-type at the permissive temperature but are completely blocked immediately after shifting ΔC30 cells to the restrictive temperature. This indicates that the primary role for the Vps15 protein kinase (and presumably the Vps34 lipid kinase) is to regulate intracellular protein sorting decisions in yeast. The Vps34 lipid kinase may be directly activated by the Vps15 protein kinase. Ligand (p2CPY) binding to a transmembrane sorting receptor in the Golgi could result in the activation of Vps15p which in turn could activate Vps34p. In such a model, Vps15p and Vps34p effectively act as components of a signal transduction complex that converts the signal received by a specific membrane receptor(s) into a second messenger (PI 3-phosphate) that could recruit and/or stabilize as yet uncharacterized effector proteins (e.g., vesicle coat proteins).

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H 022 SORTING OF SURFACE PROTEINS IN THE TRANS GOLGI NETWORK OF EPITHELIAL CELLS AND NEURONS,
Kai Simons, Carlos Dotti, Paul Dupree, Lukas Huber, Teymuras Kurzchalia and Robert Parton, European Molecular Biology Laboratory, Heidelberg, Germany.

In polarized MDCK cells, apical and basolateral proteins are sorted into separate vesicular carriers in the trans Golgi network before delivery to the appropriate membrane domains. We are using several strategies to dissect the molecular machinery involved in the vesicular budding and fusion events. In order to identify proteins interacting with sorting machinery, we have solubilized Golgi-derived transport vesicles with the detergent CHAPS and showed that an apical marker, influenza haemagglutinin (HA), formed a large complex together with several integral-membrane proteins. Remarkably, a similar set of CHAPS-insoluble proteins was found after solubilisation of a total cellular membrane fraction. A cDNA encoding one protein of this complex, VIP21 (Vesicular Integral-membrane Protein of 21KDa), was cloned and sequenced. The protein was localised mainly on the Golgi-apparatus, vesicular structures and on the cell surface. Interestingly, VIP21 was preferentially localized to caveolae on the plasma membrane. VIP21 may be a component of the molecular machinery involved in processes of vesicular transport. Another approach has been the search for small GTP binding proteins involved in the transport events. One such protein, rab8, has been identified in the basolateral vesicles. This protein is also found in the dendrites of neurons (but excluded from axons) and in BHK cells. These results support the hypothesis that the basolateral and dendritic plasma membrane domain are equivalent to the constitutive pathway from the Golgi complex to the cell surface.

H 023 MECHANISMS OF MEMBRANE PROTEIN SORTING IN YEAST, Steven F. Nothwehr, Christopher J. Roberts, and Tom H. Stevens, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.

The sorting signals of two type II membrane-bound dipeptidyl aminopeptidases (DPAPs) were analyzed in the yeast *Saccharomyces cerevisiae* (1). Removal or substitution of either the cytoplasmic or transmembrane domain of DPAP B, a vacuolar membrane protein, did not affect its transport to the vacuolar membrane. Substitution of the DPAP B transmembrane domain with the membrane spanning domain from a non-vacuolar protein also did not prevent delivery of this hybrid protein to the vacuole. Like wild type DPAP B, the delivery of the DPAP B hybrid proteins (carrying deletions or substituted domains) to the vacuole was unaffected in the secretory vesicle-blocked *sec1*-ts yeast mutant; thus, transport to the vacuolar membrane was not via the plasma membrane followed by endocytosis. Several different models can explain these data, including a simple model in which the vacuolar membrane, not the plasma membrane, is the default compartment for membrane proteins of the yeast secretory pathway.
To distinguish among various models, we analyzed the retention signal of the yeast Golgi membrane protein DPAP A. A fusion protein containing the cytoplasmic domain of DPAP A and the transmembrane and luminal domains of DPAP B (A-B-B) was localized exclusively to the Golgi apparatus, demonstrating that the 118 residue cytoplasmic domain of DPAP A is sufficient for Golgi retention. DPAP A became mislocalized to the vacuolar membrane either when it was overexpressed or when mutations were introduced into its cytoplasmic domain. Substitution of the DPAP A transmembrane domain with a synthethic hydrophobic sequence ($X=L[LALV]_5$) did not affect retention of the intact hybrid protein (A-X-A) or vacuolar delivery of the retention-defective form Δ-A-X-A, indicating that the DPAP A transmembrane domain is not involved in either retention or targeting. In all cases, delivery of these membrane proteins to the vacuole did not involve transient appearance at the plasma membrane followed by endocytosis to the vacuole (1). These data are consistent with a model in which membrane proteins are delivered to the vacuole along a default pathway. Also consistent with the vacuolar default model is the observation that retention-defective forms of two other yeast Golgi membrane proteins, Kex2p (2) and Kex1p (3), are also transported to the vacuolar membrane.
To further characterize the Golgi retention signal in the DPAP A cytoplasmic domain we subjected this region to mutational analysis. Deletion analysis indicated that the most important elements for Golgi retention were within an eight residue stretch containing two Phe residues. Alteration of either Phe residue eliminated Golgi retention and resulted in delivery of the protein to the vacuolar membrane. A ten amino acid region from DPAP A including the Phe residues was sufficient for Golgi retention of alkaline phosphatase (ALP), a type II vacuolar membrane protein. The efficiency of Golgi retention via the DPAP A signal was diminished by overexpression of Kex2p, suggesting that multiple Golgi membrane proteins may be retained by a common machinery. We are currently investigating the mechanism of retention of DPAP A in the yeast Golgi, and initiating a screen for yeast mutants that mislocalize wild type DPAP A to the vacuole.

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Late Abstract

HOW DO CHAPERONES RECOGNIZE THEIR SUBSTRATES? Samuel J. Landry, Jennifer Maxwell, Teddy Scott, and Lila M. Gierasch, University of Texas Southwestern Medical Center, Dallas, TX 75235-9041.

Molecular chaperones bind only to substrate proteins that are incompetely folded. Beyond this prerequisite, they show little preference for primary structures as many substrates can bind the same chaperone. We have been investigating the basis of chaperone binding through the analysis of the mode of binding of model peptides to different types of molecular chaperone. Application of a nuclear magnetic resonance (NMR) experiment, viz. *determination of transferred nuclear Overhauser effects* (NOEs), yields a picture of the bound conformation of the model peptide. Peptides bound to the E. coli Hsp60, GroEL, often display a helical conformation, as shown by the pattern of trNOEs (1). Consideration of data for binding of many different peptides shows that helix formation is not essential. Instead, we find that the ability of peptides to bind correlates with their ability to cluster hydrophobic residues on one face of the peptide, as measured by their retention times on reversed phase high pressure liquid chromatography (HPLC) (2). In those cases studied in detail, the GroEL-bound conformation places hydrophobic residues on one face of the molecule. We conclude that the GroEL binding site is a hydrophobic surface. It is likely that ATP modulates the affinity of this hydrophobic site, and that GroES enhances the cooperativity of the ATP-mediated affinity change (3). By contrast with GroEL substrate binding, we find that peptides bound to DnaK (4) or to BiP (Landry, Blond-Elguindi, Gething, and Gierasch, unpublished) are in an extended conformation with some binding interactions involving backbone groups. This result from trNOE data is entirely consistent with others' conclusions (5) regarding protein substrates of the Hsp70 and Hsp60 families and reinforces the model in which Hsp70 binding to a nascent chain precedes Hsp60 binding.

We have also explored structural features in GroES that may be critical to its interaction with GroEL. We have found a region in GroES between residues 17 and 32 that is mobile by NMR and is adjacent to a site susceptible to cleavage by trypsin. Binding to GroEL immobilizes this region and protects it from proteolysis. Moreover, this region is of functional importance, as it harbors mutations that block maturation of lambda phage (Zeilstra-Ryalls, Fayet, & Georgopoulos, unpublished results). A peptide corresponding to this sequence binds to GroEL, and its binding involves a sequence of hydrophobic residues IVL. It is tempting to speculate that this mobile loop in GroES binds to the same sites as a substrate protein and influences the ATP-mediated GroEL conformational change by simultaneously binding all GroEL monomers.

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Protein Targeting, Secretion and Heat Shock Proteins

H 101 EFFECT OF ADENOVIRUS E1a NUCLEAR LOCALIZATION SIGNAL STRUCTURE ON THE KINETICS OF NUCLEAR TRANSLOCATION, Douglas H. Sweet[1] and Robert H. Lyons[2], Departments of Biology[1] and Biological Chemistry[2], The University of Michigan, Ann Arbor, MI 48109

Most nuclear proteins examined to date contain a short stretch of predominantly basic amino acids that is necessary for nuclear entry - a nuclear localization signal [NLS]. Understanding the relationship between NLS structure and function is an essential step toward unraveling the underlying mechanisms of nuclear translocation. Therefore, we have performed extensive point mutagenesis of the five amino acid long carboxyl terminal NLS of the adenovirus E1a protein [KRPRP] in order to observe the effect on its function. Wild type E1a protein and E1a variants were obtained by over expression in bacteria. Purified E1a proteins were then microinjected into the cytoplasm of cultured *Vero* cells. Any protein possessing an active NLS is subsequently carried into the nucleus, as determined by immunofluorescent staining. Merely by altering the signal coding region of an E1a expression plasmid, different NLS's can be tested in exactly the same context and at precisely one copy per molecule of E1a. The time required for complete translocation is taken as an index of signal strength and we have ranked a number of variant NLS's according to their strength. The E1a NLS was quite tolerant to mutation and the main effect was to alter the kinetics of translocation rather than to prevent nuclear entry altogether. In general, the rate of translocation was enhanced by any increase in the basicity of the NLS or by an increase in Lysine content (i.e. substituting Lys for Arg). Nuclear entry was severely impaired if the sole Lysine residue was mutated and, in addition, position 4 [KRPRP] must be basic for wild type signal function. We have found, contrary to earlier reports, that for E1a the second signal position does not have to be basic. Also, while the third position does not have to be basic, we note that if it is it can greatly enhance the strength of the NLS. Interestingly, when placed on E1a, the NLS from SV40 T-antigen [PPKKKRKV] is six fold stronger than the native E1a NLS. Clearly, not all nuclear transport signals are equally effective. Our results have important implications for the study of NLS receptors. Many cytosolic and nuclear pore complex proteins have been shown to bind NLS's, yet we know virtually nothing about how they interact with signals or which ones are involved in the rate limiting step of translocation. We suggest that the signal binding protein(s) involved in the rate limiting step should show appropriate affinity towards the various mutant NLS sequences described herein.

H 102 CLONING, SEQUENCING AND DISRUPTION OF *SRP54* FROM *Yarrowia lipolytica* ENCODING A HOMOLOGUE OF THE SIGNAL RECOGNITION PARTICLE 54 KILODALTON SUBUNIT, David M. Ogrydziak and In Hyung Lee, Institute of Marine Resources, University of California, Davis, CA 95616

The *SRP54* homologue from *Y. lipolytica* was cloned and sequenced in order to determine its *in vivo* role in the synthesis and secretion of secretory proteins. Degenerate primers designed based on the sequences of the *SRP54* genes from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and mouse were used in a polymerase chain reaction to synthesize a probe for isolation of the *SRP54* gene from a *Y. lipolytica* λ Charon 4 library. Sequencing of 2467 nucleotides revealed one long open reading frame of 1608 nucleotides coding for a polypeptide of 59 kilodaltons. The predicted polypeptide has 55%, 57% and 50% sequence identity to the *SRP54* homologues of *S. cerevisiae, S. pombe*, and mouse, respectively. Like these *SRP54* polypeptides, the *Y. lipolytica SRP54* homologue has two domains -- an N-terminal domain with a highly conserved GTP-binding site (G-domain) and a methionine rich C-terminal domain (M-domain). The *SRP54* coding region has been deleted/disrupted in a diploid. Preliminary results suggest that, as in *S. cerevisiae*, the *Y. lipolytica SRP54* gene is important but not essential for cell growth. If this is confirmed, then previous results indicating that at least one copy of *SCR1* or *SCR2* (coding for SRP 7S RNAs) was essential for cell growth will have to be reexamined.

H 103 CHARACTERIZATION OF SEC63p AND ASSOCIATED ER MEMBRANE PROTEINS IMPLICATED IN PROTEIN TRANSLOCATION
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SEC63 encodes an integral membrane protein required for the translocation of pre-secretory proteins into the ER. Antibody raised against a recombinante form of Sec63p co-immune precipitates Sec61p, Sec62p and two new polypeptides of 23kD and 31.5kD. Sec61p and Sec62p are also integral ER membrane proteins required for translocation. We have purified this protein complex from detergent solubilized extracts, generated peptides corresponding to the 23kD and 31.5 kD proteins, and have cloned and sequenced the genes that encode these proteins. p23 is predicted to be a cytosolic protein localized to the cytoplasmic face of the ER. The 31.5 kD protein has one predicted transmembrane domain. Neither protein shows significant sequence homology to other proteins in the data base. The role of these proteins in polypeptide translocation is being explored.

H 104 A FUNCTIONAL ASSESSMENT OF INSERTION/ DELETION POLYMORPHISMS IN THE SIGNAL PEPTIDE OF HUMAN APOLIPOPROTEIN B, Stephen L.Sturley[1], Philippa J. Talmud[2], Stephen E. Humphries[2] and Alan D. Attie[1], [1]Dept of Biochem., U.W. Madison, Madison, WI 53706 and [2]Centre for Genetics of Cardiovascular Disorders, Univ. College and Middlesex School of Medicine, Rayne Institute, 5 University St., London, U.K.

Apolipoprotein B (apoB) is the structural protein of human low and very low density lipoproteins and as such directly effects the serum levels of cholesterol and triglyceride. Genetic variation in this protein is therefore a potential risk factor for coronary heart disease. The most common allele of human apoB encodes a 26 aa signal peptide (SP) mediating entry into the secretion pathway, followed by 4536 residues of the mature protein. Two variant apoB alleles have been identified with an insertion or deletion in the hydrophobic core of the SP. In certain human populations, these polymorphisms are associated with variation in plasma concentrations of cholesterol or triglyceride. The SP polymorphisms may mediate altered synthesis and/or secretion of apoB or alternatively, are linked to a functional defect elsewhere in the gene. To test the former hypothesis, we have replaced the SP of yeast invertase, encoded by the *SUC2* gene, with the normal and variant apoB signal peptides. When introduced into *SUC2* deficient yeast strains the apoB deletion allele/*SUC2* gene fusion mediated approximately 50% lower levels of secreted invertase activity than the normal apoB/*SUC2* gene fusion. Unlike with the normal allele, intracellular accumulation of invertase activity was detected in cells carrying the deletion allele, suggesting a defect in translocation into the ER. This reduced secretion phenotype in yeast was in accordance with the reduction in serum triglyceride levels observed in patients homozygous for the deletion allele of apoB.

H 105 THREE GTP BINDING PROTEINS INTERACT TO INITIATE PROTEIN TRANSLOCATION INTO THE ENDOPLASMIC RETICULUM, Joshua D. Miller and Peter Walter, Department of Biochemistry and Biophysics, UCSF School of Medicine, San Francisco, CA 94143-0448

Signal sequence bearing proteins are targeted from the cytoplasm to the Endoplasmic Reticulum(ER) in a GTP dependent manner. This process is initiated by the Signal Recognition Particle(SRP) binding to the signal sequence of an elongating nascent chain. SRP then chaperones the signal sequence to the ER via its interaction with the heterodimeric SRP receptor(SR). The α-subunit of SR is required for binding to SRP and is anchored to the cytoplasmic face of the ER by the transmembrane, β-subunit. The signal sequence is bound to the SRP ribonucleoprotein complex through its 54kd subunit and is released when SRP54 binds to SR. Upon release, the signal sequence is free to interact with the more downstream components of the translocation apparatus. SRP, SRα and SRβ all bind GTP thus forming a chain of interacting GTP binding proteins that are anchored to the ER membrane by a novel transmembrane GTP binding protein(SRβ). To elucidate the role of GTP in the SRP·SR interaction we turned to a solubilized system using purified components. We find that SR acts to promote GTP binding and hydrolysis by SRP 54. Since in a targeting complex, SRP would be bound to the signal sequence when it interacts with SR, we tested the effect of adding synthetic signal peptides to the GTPase reaction. Interestingly, we found that peptides corresponding to functional signal sequences inhibited the GTPase at µM concentrations whereas mutant, nonfunctional signals had no effect. GTP binding assays demonstrated that signal peptides inhibited the GTPase activity by blocking GTP binding to SRP54. This result suggests that SRP54 can not bind to GTP and signal sequence simultaneously and we propose a model where SR mediated GTP exchange onto 54 regulates its release of signal sequence.

H 106 ISOLATION OF GTP-BINDING DOMAIN MUTANTS OF *SRH1* USING "DOPED" MUTAGENESIS, Amaya, Y. and Nakano, A., Department of Biochemistry, Yokohama City University School of Medicine, Yokohama 236, Japan and Department of Biology, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Saccharomyces cerevisiae SRH1 (*SRP54^{sc}*) encodes a homologue of the 54K subunit of mammalian signal recognition particle. Its N-terminal portion is also homologous to the cytosolic domain of the α subnit of the signal recognition particle receptor including consensus sequence elements for a GTP binding site. The C-terminal portion is an unusual methionine-rich domain containing several repetitive sequences. Functional analysis has shown that the *SRH1* product (Srh1p) is required for vigorous cellular growth and involved in the ER translocation of secretory proteins.

To investigate the role of GTPase activity of Srh1p on the ER translocation of secretory proteins, we have tried to isolate GTP-binding domain mutants of *SRH1*. Degenerate oligonucleotides for the coding regions of three GTP-binding consensus sequences (GXXXXGK, DXXG, TKXD) were used for site directed mutagenesis (doped mutagenesis). Galactose dependent *srh1* cells were transformed with the plasmid libraries carrying mutations at the GTP-binding site of *SRH1*. Among the 410 transformants, 2 transformants showed cold sensitive and 39 transformants showed function-less phenotypes on glucose medium. The precursor form of α-mating factor accumulates in the cold sensitive mutants under the restrictive condition. This approach will provide various mutants of *SRH1* with high efficiency.

H 107 PARTIAL PURIFICATION AND RECONSTITUTION OF AN ATP-TRANSPORTER FROM DOG PANCREAS MICROSOMES AND ITS ROLE IN PREPROTEIN TRANSLOCATION, Peter Mayinger and David I. Meyer, Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024

We have recently characterized an ATP uptake system in yeast ER-derived vesicles which functions in preprotein translocation by supplying a luminal factor with ATP. Here we provide evidence that ATP transport into dog pancreas microsomes is also a prerequisite for translocation in this system. Inhibition of ATP uptake into dog pancreas microsomes by DIDS, a potent inhibitor of the mammalian ATP transporter, effectively blocks preprotein translocation. The DIDS inhibition of translocation could be prevented by coincubation with ATP which provides further evidence that ATP transport is connected to preprotein translocation. To investigate ATP transport into mammalian microsomes in greater detail, a partially-purified detergent extract of dog pancreas microsomal membranes was functionally reconstituted into proteoliposomes. This system was used for kinetic characterization of the microsomal ATP transporter. The reconstitution of the ATP transporter will be combined with existing procedures for reconstitution of mammalian preprotein translocation in order to define the role of ATP transport in mammalian preprotein translocation.

H 108 THE ROLE OF ATP IN PROTEIN TRANSPORT INTO MAMMALIAN MICROSOMES, Peter Klappa, Richard Zimmermann and Thomas Dierks, Institut für Biochemie II, Universität Göttingen, D-3400 Göttingen, FRG

Ribonucleoparticle (i.e. ribosome and SRP)-independent transport of proteins into mammalian microsomes is mediated by ATP and molecular chaperones such as Hsc70. A chemically synthesized presecretory protein, after solubilization in DMSO and subsequent dilution into an aqueous buffer, was processed by and transported into mammalian microsomes even in the absence of the molecular chaperones. Membrane insertion under these conditions depended on the hydrolysis of ATP and involved a microsomal protein which could be photoaffinity inactivated with azido-ATP. The same microsomal protein with a sensitivity towards photoaffinity modification with azido-ATP was observed to be involved also in ribonucleoparticle-dependent transport. Therefore we suggest that a microsomal protein depending on ATP-hydrolysis is engaged in membrane insertion of both, ribonucleoparticle-dependent and -independent precursor proteins. Recent experiments with a proteoliposomal system allow us to exclude most of the lumenal proteins, such as BiP, as being responsible for this ATP-dependent and azido-ATP sensitive step. By using this more defined system (i.e. a purified precursor protein and proteoliposomes) we are currently trying to identify and characterize this ATP-hydrolyzing protein.

H 109 A NOVEL YEAST GENE FUNCTIONING IN PROTEIN TRANSLOCATION, *SSS1*, ENCODES A MEMBRANE PROTEIN, F. Képès, Y. Esnault and M.-O. Blondel, Service de Biochimie et de Génétique Moléculaire, Bât.142, CE/S, 91191 Gif-sur-Yvette Cedex, France

How does a polar protein cross a lipidic and continuous membrane ? An instance of such a phenomenon is provided in eukaryotes by the translocation of polypeptides from their site of synthesis, the cytoplasm, to the lumen of the endoplasmic reticulum (ER). We have approached this process in *Saccharomyces cerevisiae* through the study of a gene, *SSS1*, that restores at elevated copy number a wild-type phenotype in the translocation mutant *sec61*. *SSS1* is an essential gene that encodes a 8,900 dalton basic protein (Sss1p). Although most of Sss1p is hydrophilic, with only two short hydrophobic stretches close to its carboxy-terminus, it fractionates after gentle cell lysis in a 10,000*g* pellet. This association with a rapidly sedimenting material is resistant to treatments with high salt, urea or hydroxylamine. Dissociation occurs in presence of 0.5% Triton X-100. This behavior indicates that Sss1p is a membrane protein. Analysis of the vicinity of Sss1p by chemical crosslinking with bifunctional reagents and immunoprecipitation has revealed that this protein is in close spatial proximity to the ER components of the translocation machinery encoded by *SEC61*, *SEC62* and *SEC63*. Precursor forms of secretory proteins start to accumulate within the cells 2-4 hours after repression of Sss1p synthesis in a conditional expression mutant. This phenotype indicates that *SSS1* functions in preprotein translocation to the ER.

H 110 THE SIGNAL RECOGNITION PARTICLE IN *SACCHAROMYCES CEREVISIAE*
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Protein targeting to the endoplasmic reticulum is catalyzed by the signal recognition particle (SRP). We have identified the SRP homologue in *S. cerevisiae* and characterized its function in vivo. *S. cerevisiae* SRP is a 16S particle which includes a homologue of the signal sequence binding protein subunit of SRP (SRP54p), the *SEC65* gene product (Sec65p, which is homologous to SRP19) and a small cytoplasmic RNA (scR1). Genetic and biochemical studies suggest that each of these components is required for SRP's function in vivo and implicate an interaction between SRP54p and Sec65p. Our progress toward a purification of the yeast SRP and the developement of functional in vitro assays will be reported.

H 111 ANALYSIS OF ER LOCALIZATION SIGNALS IN BARLEY ALEURONE CELLS, Karin M.C. Sinjorgo[1], Martien P.M. Caspers[1] and Jürgen Denecke2, Dept. Mol. Plant Biotechn., Center for Phytotechnology RUL-TNO, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands[1]; Dept. Mol. Genetics, Uppsala Genetic Center, Swedish University of Agricultural Sciences, S0750 07 Uppsala, Sweden[2].
The aleurone layer of cereal grains is one of the few plant tissues which exhibit a primary function in protein secretion. Using barley aleurone protoplasts, we developed a transient expression system to investigate the transport of proteins within the plant endomembrane system. The model system was validated by demonstrating that chimera of the bacterial cytoplasmic enzyme phosphinotricin acetyl transferase (PAT) and a signal sequence, were secreted by default.
To extend the current knowledge about plant ER localization signals (Denecke, J., De Rycke, R. and Botterman, J. (1992) EMBO J. 11, 2345-2355) to monocotyledonous plant species, we investigated the specificity of the ER retention machinery in our model system. When one of the tetrapeptides KDEL, HDEL or RDEL were added to the C-terminus of PAT, the resulting protein chimera was retained by the monocot. However, HDEL was found to constitute the most efficient signal in aleurone protoplasts, whereas no difference between the three chimera was observed in leaf protoplasts from dicots. Possibly, small differences in affinity of the salvage receptor for the test ligands only become apparent in the barley aleurone cells, which exhibit a high activity of endomembrane traffic. Alternatively, the salvage receptor in the aleurone of the monocot barley might have a slightly modified retention specificity compared to the receptor in dicots. In both systems, the ER-retention machinery proved to be highly specific. KDDL, KDEI and KDEV were not recognized and PAT-KEEL showed only partial retention. However, whereas PAT-SDEL was secreted by tobacco protoplasts, barley aleurone cells were found to retain this chimera to some extend. Experiments are currently being carried out to determine possible differences in retention specificity in more detail.

H 112 *IN VIVO* FOLDING OF THE HUMAN ASIALO-GLYCOPROTEIN RECEPTOR H1: FOLDING INTERMEDIATES AND THE EFFECT OF REDOX STATE, Rebecca G. Wells and Harvey F. Lodish, The Whitehead Institute for Biomedical Research, Cambridge, MA 02142
The human asialoglycoprotein receptor H1 is a type II membrane glycoprotein which is a member of the C-type or calcium-dependent family of animal lectins, binding specifically to galactose residues. It contains four disulfide bonds, two in the stem domain and two in the lectin-binding domain. In Hep G2 cells, two disulfide bonding intermediates have been resolved; folding is reversibly inhibited by the addition of reducing agents or calcium ionophores to the cells (H.F. Lodish, N. Kong, & L. Wikstrom, *J. Biol. Chem.* **267**, 12753 (1992)).
We have developed an *in vitro* translation/canine pancreatic microsome system which we have used to study further the folding of H1. H1 folds in this system through disulfide bonding intermediates demonstrable via non-reducing SDS-PAGE. We have altered the redox ratio of glutathione, the major ER redox buffer (C. Hwang, A.J. Sinskey, & H.F. Lodish, *Science* **257**, 1496 (1992)), and have determined that folding is dependent on a defined and (compared to the cytoplasm) relatively oxidized redox state; a more reduced state results in the accumulation of folding intermediates. Soluble recombinant forms of H1 (without the cytoplasmic or transmembrane regions) which include either the stem plus lectin-binding domain (with four disulfide bonds) or the lectin-binding domain (with two disulfide bonds) only also fold in this system with demonstrable disulfide bonding intermediates on non-reducing gels; they show a similar response to changes in the redox state. These soluble proteins can be transiently expressed in COS cells where they are secreted in the folded state and show similar folding intermediates.

H 113 THE ER-LOCALIZED MOLECULAR CHAPERON BiP IN TOMATO. David J. Meyer and Alan B. Bennett. Department of Vegetable Crops, University of California, Davis, CA 95616.

It has been proposed (Ellis, R.J. *Nature* 345:484) that the ability to manipulate gene expression in a productive manner may require an understanding of the roles of molecular chaperones in the expression of functional gene products. In addition, relatively little is known about the mechanisms of protein traffic through the ER of plants. We have initiated studies of binding protein (BiP) in higher plants; we have isolated and sequenced cDNA and genomic DNA clones which correspond to the single tomato BiP gene. Tomato BiP shows high sequence identity to other BiP genes and to other hsp70s, and the DNA encodes the C-terminal sequence -H-D-E-L-COOH. Polyclonal antibodies directed against the C-terminal 72 amino acids encoded by the cDNA recognize a 75 kD protein that is greatly enriched in the ER. N-terminal sequencing of this enzyme purified from suspension-cultured tomato cells allowed determination of the signal sequence cleavage site. PCR mutagenesis was used to construct a mutant cDNA encoding tomato BiP lacking the signal sequence for expression in *E. coli*. In order to allow high-level expression in *E. coli*, the mutant BiP gene was expressed in a T_7 RNA polymerase-based expression system (Studier, F.W., *et al. Meth. Enzymol.* 185:60). The overexpressed protein has been purified by hydrophobic interaction and ion exchange chromatography to >95% purity; this enzyme exhibits ATPase activity with properties similar to mammalian BiP (Kassenbrock and Kelly, *EMBO* 8:1461-7). This system will allow further characterization of BiP enzymatic activity through site-directed mutagenesis and the purification of sufficient quantities of enzyme for both structural studies and the identification of co-chaperones and substrates of BiP in the ER.

H 114 GRP94, AN ER STRESS PROTEIN, ASSOCIATES WITH BiP AND UNASSEMBLED IMMUNOGLOBULIN CHAINS. J.R. Melnick, S. Aviel, and Y. Argon, Department of Immunology, Duke University Medical Center, Durham, NC, 27710

It is well established that the molecular chaperone BiP/GRP78 associates in the ER with both secretable and non-secretable polypeptides; unassembled immunoglobulin (Ig) heavy (H) and light (L) chains are prominent examples. Using a thiol-cleavable cross-linker, DSP, we now show that another ER stress protein, GRP94/ERp99, is co-precipitated with H chains and non-secretable L chains. In contrast, little GRP94 is associated with secretable L chains. We also detect very little GRP94 in association with assembled Ig molecules, strongly suggesting that GRP94 assists in the folding or assembly of newly synthesized polypeptides. Studies are under way to determine whether GRP94, like BiP, associates transiently with secretable Ig chains. GRP94 associates with Ig chains expressed in transfected COS fibroblasts as well as murine myelomas, suggesting that its role in protein maturation is not limited to immunoglobulins. We demonstrate the presence of complexes consisting of all three molecules: Ig chain, BiP, and GRP94. Moreover, BiP and GRP94 associate even in the absence of Ig chains. The presence of both GRP94 and BiP in the same complex with unassembled Ig chains suggests that these two stress proteins work in concert as molecular chaperones.

H 115 ASSOCIATIONS OF HSP70 WITH A SUBSET OF DORSAL ROOT GANGLIA (DRG) AND SCIATIC NERVE (SN) PROTEINS, Bruce Tedeschi and Richard P. Ciavarra, Departments of Anatomy & Neurobiology and Microbiology $ Immunology, Eastern Virginia Medical School, Norfolk, VA 23501

Neurons represent good model systems for investigations of protein transport and cell compartmentalization since they display an axonal cytoplasmic specialization devoid of translational machinery. We utilized a monoclonal antibody to HSP70 to immunoprecipitate radiolabelled DRG and SN proteins and subsequently analyzed the immunoprecipitates by 1 and 2 dimensional polyacrylamide gel electrophoresis. Results showed that about 20-30 DRG polypeptides co-immunoprecipitated with HSP70. Most of the immunprecipitated proteins are unidentified but actin, tubulin, and probably the 200kDa neurofilament subunit were among the immunoprecipitated proteins. The monoclonal antibody to HSP70 also immunoprecipitated a similar profile of proteins in SN. We speculate that HSP70 may play multiple roles in the assembly, transport, and compartmentalization of neuronal proteins.

H 116 STUDIES ON EUKARYOTIC HSP70 MOLECULES USING DOMINANT NEGATIVE MUTATIONS IN HOMOLOGOUS AND HETEROLOGOUS IN VIVO SYSTEMS, Peter J. Murray and Richard A. Young, Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge MA 02142

Eukaryotic HSP70 molecules appear to function to ensure the correct folding of certain polypeptide substrates in vivo as well as complexing denatured proteins and assisting translocation of polypeptides across membranes. HSP70s in eukaryotic cells can be considered to fall into two groups: cytoplasmic forms which have overlapping, degenerate functions and organellar forms which are non-degenerate. The latter group has two members: KAR2/BiP present in the endoplasmic reticulum and the mitochondrial matrix HSP70. Both of these proteins are essential for viability in yeast.. To study both systems we are using the approaches of constructing dominant negative alleles and epitope tagging to be able to differentiate specific HSP70-substrate interactions. Two HSP70s are being studied: mouse HSP70.1(a cytoplasmic HSP70 encoded by the major histocompatability locus) and yeast and human KAR2/BiP. Each has been epitope tagged and expression studies have been conducted in a variety of cell types including yeast, mouse fibroblasts and mouse and human haemopoetic cells. We are attempting to isolate dominant negative mutations in each by using negative selection screens in yeast. Recent results of such screens shall be presented.

H 117 ACTIVATION OF HUMAN HEAT SHOCK GENES IS ACCOMPANIED BY OLIGOMERIZATION, MODIFICATION AND TRANSLOCATION OF HEAT SHOCK TRANSCRIPTION FACTOR HSF1,
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Transcriptional activity of heat shock (hsp) genes is controlled by arrays of inverted repeats of the element NGAAN. To date genes for two human factors, HSF1 and HSF2, have been isolated. To define their properties as well as the changes they undergo during heat stress activation, polyclonal antibodies were prepared to these factors. Using these tools we have shown that human HeLa cells constitutively synthesize HSF1 but did not detect HSF2. In unstressed cells HSF1 is present in complexes of about 400 and 200 kDa, resepectively, unable to bind DNA. Heat treatment enables the DNA binding function of HSF1 and induces a concomitant shift in apparent molecular size to about 700 kDA, consistent with hexamerization of the molecule. Human HSF1 expressed in *Xenopus* oocytes does not bind DNA, but derepression of the DNA binding function of HSF1 as well as conversion of smaller to 700 kDa HSF1 containing complexes is prompted by heat treatment at the same temperature at which hsp gene expression is induced in this organism, indicating that the latter feature is inherent in the structure of human HSF1, and that derepression of its DNA binding function may only require highly conserved cofactors. Inactive HSF1 resides in the cytoplasm of human cells, upon activation rapidly translocates to a soluble nuclear fraction and shortly thereafter, becomes associated with the nuclear pellet. Unstressed cells contain both apparently unmodified and modified forms of HSF1. Upon heat shock the modified forms translocate to the nucleus undergoing further modification. These different processes provide multiple points of regulation of hsp gene expression some of which may involve the control of protein-protein interactions between HSF1 and chaperon molecules.

H 118 HSC70/TOPOISOMERASE INTERACTIONS DURING HEAT STRESS: EVIDENCE THAT PROTECTION AND RECOVERY OF CATALYTIC ACTIVITY IS ATP-INDEPENDENT.
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T cells rendered thermotolerant by a prior modest heat shock reinitiate DNA synthesis following a severe heat-shock challenge more rapidly than control cells. Recovery of DNA synthetic capacity is related, at least in part, to protection of nuclear type I topoisomerase activity. Interestingly, heat-inactivated topoisomerase activity in both thermotolerant and control cells returns to normal levels following an incubation at a non-stress temperature. We have speculated that during heat stress hsp70 proteins migrate into the nucleolus where they associate with type I topoisomerase to either limit thermal injury and/or accelerate recovery of protein function. This view is now supported by our observations that immunoprecipitation of the 70 kDa heat shock cognate protein (hsc70) from heat-shocked cell lysates co-purifies type I topoisomerase. In the absence of exogenous ATP, purified hsc70 but not actin protected topoisomerase activity from heat-inactivation *in vitro*. The addition of hsc70 to previously heat-denatured type I topoisomerase also resulted in reactivation of the enzyme. However, reactivation of heat-denatured topoisomerase by hsc70 was inefficient relative to a cell lysate which also reactivated heat-denatured topoisomerase in an ATP-independent fashion. These studies suggest that 1) protection and restoration of topoisomerase activity by purified hsc70 or a cell lysate does not require ATP, 2) hsc70 may require other cellular factors for optimal protection of protein function and/or 3) the cell contains another protein folding pathway which is independent of hsc70 and ATP.

H 119 CYTOSOLIC REACTIONS IN ORGANELLE BIOGENESIS: REGULATION OF HSP70 ACTIVITY BY AN EUKARYOTIC DNAJ HOMOLOG.
D.M. CYR, A. J. Caplan and M.G. Douglas, Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill. N.C. 27599-7260.USA.
Cytosolic Hsp70 molecules and unidentified cytosolic proteins participate in the maintenance of pre-proteins in transport competent conformations. Insight into the identity these proteins comes from recent genetic and biochemical studies in procaryotes which demonstrate that Hsp70 (DnaK) functionally interacts with two other cytosolic heat shock proteins, DnaJ and GrpE. Several eukaryotic DnaJ homologs have recently been identified. To characterize interactions between eukaryotic Hsp70 and DnaJ homologs, cytosolic yeast Hsp70 (SSA1p) and DnaJ (YDJ1p) homologs were purified. The influence of YDJ1p on SSA1p ATPase activity and polypeptide substrate binding were then tested. SSA1p hydrolyzed ATP at 2-5 nmol/mg/min. YDJ1p had no detectable ATPase activity. However, YDJ1p, at a 1:1 molar ratio, stimulated ATP hydrolysis by SSA1p 8-10 fold. Interactions between YDJ1p and SSA1p appeared specific since neither unfolded polypeptides nor other folded proteins stimulated SSA1p ATPase activity. Additionally, YDJ1p had no effect on the ATPase activity of purified BiP or DnaK. ATP hydrolysis has been correlated with release of bound polypeptides from Hsp70. Analysis on native gels indicated that SSA1p formed a stable complex with ^{125}I-F1β presequence peptide and ^{125}I-carboxymethylated lactalbumin. ATP alone had little effect on complex formation, however, ATP in combination with YDJ1p reduced complex formation by as much as 80%. YDJ1p alone or in combination with AMP-PNP had no effect on complex formation. This indicated that stimulation of SSA1p ATPase activity by YDJ1p was responsible for reductions in complex formation. Side by side comparison of substrate binding to SSA1p, BiP and DnaK indicated SSA1p was unique among these Hsp70 homologs. Significant reductions in F1β presequence peptide binding to BiP and DnaK, but not SSA1p, were observed in the presence of ATP. These differences may reflect a specialized mechanism for SSA1p function which facilitates the stabilization of pre-proteins in transport competent conformations prior to import. In this mechanism interaction of SSA1p with YDJ1p would facilitate release of pre-proteins from SSA1p and their entrance into protein transport pathways. *In vivo* studies which demonstrate that temperature sensitive mutations in YDJ1p cause defects in protein import into both mitochondria and the endoplasmic reticulum at the non-permissive temperature support this proposal.
Supported by grants AHA NC-90-4 to DMC and NIH R01-36537 to MGD.

H 120 EFFICIENT SECRETION OF THE HUMAN FACTOR VIII LIGHT CHAIN FROM BACULOVIRUS-INFECTED INSECT CELLS.
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Factor VIII is a large, complex plasma glycoprotein involved in the process of blood coagulation. The protein consists of a heavy chain (200kDa) and light chain (80kDa) which assemble into a stable complex mediated by metal ions, in the presence of von Willebrand factor. The production of recombinant factor VIII from cDNA constructs transfected into mammalian cells has proven to be an inefficient process, due in part to the retention of protein within the endoplasmic reticulum. To investigate the secretory ability of each chain we have examined expression and secretion in insect cells, of a B domain-deleted heavy chain cDNA construct and a light chain construct engineered to contain the factor VIII signal sequence. While the light chain appeared to be expressed and secreted efficiently using a baculovirus vector, the truncated heavy chain gave low levels of expression and was undetectable in cell medium. The levels of secreted light chain are around 10 fold higher than those obtained with a B domain-deleted heavy chain and light chain fusion construct expressed in the same system. These data suggest that the light chain is more efficiently secreted than heavy chain, which may have implications for the mechanism of native factor VIII secretion.

H 121 A CONSERVED SURFACE PATCH ON THE SURFACE OF LIGHT CHAIN VARIABLE DOMAIN FORMS A STRUCTURE NECESSARY FOR IMMUNOGLOBULIN SECRETION. Sigal Aviel and Yair Argon, Department of Immunology, Duke University Medical Center, Durham, NC, 27710

Immunoglobulin light chains are normally expressed and secreted by B cells independently of heavy chains. We have previously shown that a single point mutation on the surface of λI light chain is sufficient to block its secretion without destroying the function of the protein. We now describe a set of mutants which alter seven more conserved residues on the same surface of the variable domain of λI chain. The mutants accumulate in the ER as determined by immunofluorescence and by their association with the ER stress proteins BiP/GRP78 and GRP94/ERP99. When the non-secreted mutants are co-expressed with wild-type heavy chains, they assemble into tetrameric antibodies capable of binding antigen and are no longer associated with BiP or GRP94 indicating that they are not grossly misfolded. However, the non-secreted phenotype is dominant as these mutant immunoglobulin molecules are still arrested in the ER. Although located on three non-contiguous stretches of the polypeptide, the residues described here form a conformational surface patch on the folded protein. Our findings suggest that the precise nature of the side chains exposed to the solvent is less important for the secretion of λI than the local structure formed by the polypeptide backbone. The dominance of the non-secreted phenotype after assembly with H chain and dissociation from known molecular chaperones indicates that another protein- protein interaction mast take place before functional Ig can exit the ER.

H 122 Variable Region Domain Switches in an IgG_1. The Dominant Role of the Light Chain for Intact Immunoglobulin Secretion, C.R. Ill*, E. King, S. Cheng, L. Meyers, K. Beidler, J. Gonzales and J. Corvalan. *Protein Engineering, Imaging and Therapy Research and Development, Hybritech Inc., San Diego, CA. 92121

Immunoglobulin heavy and light chain genes were engineered to contain the opposite variable region so as to achieve expression of a V_{gamma}-C_{kappa} chain and a V_{kappa}-C_{gamma} chain. The engineering was carried out to construct a kappa dimer with anti-CEA activity and to understand certain features of the immunoglobulin secretory pathway. The V_{gamma}- C_{kappa} construct was expressed but not secreted by P3x653 myeloma or SP2/0 hybridoma cells. When an unmodified light chain was transfected into cell lines expressing the V_{gamma}- C_{kappa} chain, it would now combine with the normal light chain and be secreted as a kappa dimer. The V_{gamma}- C_{kappa}•V_{kappa}-C_{kappa} dimer had similar affinity for CEA as the Fab' from the parent IgG_1. A V_{kappa}-C_{gamma} construct was also expressed but not secreted in SP2/0 cells unless a normal light chain was transfected into the same cells to rescue this chain. The V_{kappa}-C_{gamma}•V_{kappa}-C_{kappa} immunoglobulin was secreted as a 160 kilodalton protein without affinity for CEA. Taken together these results support the role of the light chain variable region and constant region being necessary for intact immunoglobulin secretion. Our data corroborates data from others and suggests that the kappa constant region interaction with the CH-1 domain of the heavy chain is necessary for heavy chain release from BiP while the kappa variable region is necessary for secretion of the intact IgG_1 at subsequent steps in the immunoglobulin secretory pathway.

H 123 HIGH LEVEL SECRETION OF RECOMBINANT HUMAN FIBRINOGEN IN BHK CELLS IS LIMITED BY A POST-TRANSCRIPTIONAL PROCESS
Donna Prunkard and Don Foster
ZymoGenetics, Inc., Seattle, Washington.

Fibrinogen is a plasma protein which is secreted by the liver as a multimer composed of two each of three different polypeptide chains, α, β and γ. Human liver HepG2 cells are known to accumulate intracellular pools of the α and γ chains, whereas significant intracellular levels of free β chain are not detected. Based on pulse-chase data, the β chain is believed to interact with either the α or γ chain immediately following β chain transport into the ER. When recombinant fibrinogen is expressed at low levels in BHK (baby hamster kidney) cells, intracellular accumulation patterns are similar to those observed with HepG2 cells. However, when the heterologous fibrinogen genes are amplified to high levels in BHK cells, fibrinogen protein assembly and secretion are not significantly increased. Southern analysis confirms the increases in gene copy number. Northern analysis demonstrates an increase in the mRNA levels of all three chains. However, these increases are not reflected in an increase in the amount of correctly assembled multimer present in the secretory pathway or secreted into the medium. The size of the α and γ chain pools increases sigificantly, while beta chain continues to be limiting. In addition, surplus α and γ chains appear in a discrete complex which is present intracellularly as well as efficiently secreted. These results suggest the existence of tissue-specific synthesis, transport, or assembly mechanisms in liver-derived HepG2 cells which are not present in BHK cells.

H 124 **ELUCIDATING THE DOMAIN STRUCTURE OF E. COLI SECA: A STABLE 64 KILODALTON CHYMOTRYPSIN FRAGMENT POSSESSES ELEVATED ATPASE ACTIVITY IN THE ABSENCE OF ACIDIC PHOSPHOLIPIDS.** Terry L. Triplett and Lila M. Gierasch; Departments of Pharmacology and Biochemistry, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Tx 75235-9041.

SecA is a 102 kd cytosolic and peripheral inner membrane protein which is an essential component of Sec-mediated protein export in *E. coli*. SecA possesses an ATPase activity that is partially stimulated by the binding and insertion of SecA into phospholipid bilayers containing acidic phospholipids (Lill et al., *Cell* **60**, 271-280, 1990).
The mechanism for how binding to lipids activates the SecA ATPase and facilitates translocation is not understood. We have used limited proteolysis to show that a 64kd region of SecA (SecA64) is stable against digestion by chymotrypsin in the presence of ATP and magnesium ions. Stabilization is supported by ATP, ADP, ATP-γ-S, AMP-PNP and to a lesser extent by CTP and UTP, but not by AMP or GTP, in agreement with published observations describing SecA-nucleotide interactions (Shinkai, et al., J. Biol. Chem. **266**, 5827-5833, 1991). N-terminal sequence analysis of this fragment electroblotted onto PDVF membranes shows that it corresponds to the N-terminal region of SecA, and circular dichroism measurements show that it is in a folded state with defined secondary structure resembling that of native SecA. We propose that this proteolytic fragment represents a functional and structural domain of SecA. Samples of SecA digested for varying times with chymotrypsin show marked increases in ATPase activity in the absence of phospholipids in parallel with the appearance of SecA64 on SDS gels. Thus, proteolytic cleavage relieves an apparent inhibition of ATPase activity normally at work in native SecA. Assaying of the same samples in the presence of 3:1 DOPC:DOPG SUV's shows that lipid stimulation of the ATPase decreases as the uncleaved SecA is depleted, suggesting that the ATPase activity of the 64 kd fragment is no longer sensitive to lipid. This argument is supported by the observation that the ATPase activity of 95% pure SecA64 alone is higher than that of an equimolar amount of native SecA and is not stimulated by acidic lipids. On the basis of these data, we propose that the activation of SecA ATPase upon lipid binding is due to the insertion of the C-terminal region of the protein into the bilayer, relieving by some as yet unknown mechanism the inhibition of the ATPase activity residing in the N-terminal domain.

H 125 ISOLATION AND ANALYSIS OF NOVEL MUTANTS OF *E. coli prlA* (*secY*), Mary K. Olsen, Everett L. Rosey[1] and Che-Shen C. Tomich, Molecular Biology and [1]Animal Health, The Upjohn Company, Kalamazoo, MI 49007

Plasmid libraries of *prlA* (*secY*) mutations containing single basepair changes throughout the gene were generated by *in vitro* random mutagenesis. From these libraries, novel *prlA* mutants were selected for their ability to suppress the secretion defect of *E. coli* LamB in a strain with a *lamB* signal sequence deletion. Additional site-specific substitutions suggest that the nature of the amino acid residues at positions 407 and 408 is important in the structure and function of PrlA.

These *prlA* mutant libraries are being used to screen for PrlA mutations that enhance the secretion of porcine insulin-like growth factor. Enhanced secretion of IGF is screened by increased resistance to β-lactamase in the fusion of IGF to β-lactamase.

H 126 SecA, THE ATPase SUBUNIT OF THE BACTERIAL PREPROTEIN *TRANSLOCASE* ACTS AS A DIMER, Arnold J. M. Driessen and Wieny Kuiper, Department of Microbiology, University of Groningen, Haren, Netherlands.
Precursor protein translocation across the *Escherichia coli* cytoplasmic membrane requires a protonmotive-force, ATP, and *translocase*[1]. *Translocase* is a multisubunit membrane protein with a peripheral ATPase domain (SecA) and a membrane embedded domain (SecY/E). In solution, SecA exists as a homodimer[2]. The functional role of this quaternary structure is unknown. SecA denatured in 6 M guanidinium ·HCl can be renatured to a functional dimer[2]. Chemical crosslinking, size-exclusion chromatography, intrinsic tryptophane fluorescence and circular dichroism studies indicate that the denatured SecA protein refolds into its native quaternary, tertiary and secondary structure. Functional SecA heterodimer were formed of which the individual subunits were tagged with fluorescent dyes to allow measurements of the association state of the monomers by fluorescence energy transfer. SecA retained its dimeric structure during translocation, while energy-transfer was abolished only by denaturation and limited proteinase digestion. Heterodimers composed of native and 8-azido-ATP-inactivated SecA lost the ability to support translocation. It is concluded that the dimeric structure is required for functionality. These results are consistent with the observed abundance of dominant *secA* mutations[3,4].

[1]Wickner, W., Driessen, A.J.M., Hartl, F,-U. (1991) *Annu. Rev. Biochem.* **60**, 101-124
[2]Akita, M., Shinkai, A., Matsuyama, S.-I, Mizushima, S. (1991) *Biochem. Biophys. Res. Commun.* **174**, 211-216.
[3]Jasorik, G.P., Oliver, D.B. (1991) *J. Bacteriol.* **173**, 860-868.
[4]Klose, M., Schimp, K.-L., van der Wolk, J., Driessen, A.J.M., Freudl, R., (1992) submitted.

H 127 ALTERATIONS IN THE CENTRAL REGION OF M13 PROCOAT DETERMINE SEC-REQUIREMENTS. Stephanie Hugenschmidt, Ross Dalbey*, and Andreas Kuhn Dept. of Applied Microbiology, University of Karlsruhe, 75 Karlsruhe, Germany and*Dept. of Chemistry, Ohio State University, Columbus, Ohio 43210.

The membrane insertion of most exported proteins requires the bacterial translocation apparatus encoded by the *sec*-genes. Only a few proteins, among them M13 procoat, insert into the membrane in the absence of functional SecA and SecY. Previous studies of M13 procoat - proOmpA hybrid proteins showed that the mature region rather than the leader sequence determines the requirement for SecA and SecY. Alterations in the extracellular domain of M13 procoat make its membrane insertion dependent on SecA and SecY. We have systematically increased the length of this extracellular domain by adding OmpA-derived sequences of 20, 40, 60, and 80 amino acid residues. We found that the mutant with 20 additional residues is translocated *sec*-independent, although very inefficiently. The mutants with 60 or 80 additional residues inserted into the membrane only in the presence of functional SecA. The mutant with 40 additional residues was not processed by leader peptidase, suggesting that it fails to insert at all. However, we found that protease added to the outside of the membrane digested about 50% of the protein resulting in a protease resistent fragment of 6 kD. Possibly, this partially translocated and not processed species does not properly interact with the Sec components and is therefore stuck in the translocation process.

H 128 SecN, A NOVEL FACTOR COMPRISING THE PROTEIN TRANSLOCATION MACHINERY OF E. COLI, Ken-ichi Nishiyama, Shoji Mizushima, and Hajime Tokuda, Institute of Applied Microbiology, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, Japan.
We have reported that the protein translocation machinery of E. coli can be reconstituted from purified SecA, SecE, and SecY[1]. Activity of the reconstituted proteoliposomes was, however, considerably lower than that of intact everted membrane vesicles. Neither SecD nor SecF was found to enhance the activity of proteoliposomes[2]. On the other hand, we found a novel factor which remarkably enhanced translocation activity of proteoliposomes. The activity of proteoliposomes reconstituted with this factor was more than 10-fold higher than that of proteoliposomes reconstituted without this factor. The factor was recovered in soluble fraction when the octylglucoside extract of the E. coli membrane was treated with TCA. The factor lost its activity upon treatment with trypsin, indicating that the factor is proteinaceous. None of the known Sec proteins was present in the TCA supernatant fraction. The factor, named SecN, was purified. The molecular mass of SecN was found to be about 6 kDa. Roles of SecN in protein translocation will be discussed.

1) Akimaru, J., Matsuyama, S., Tokuda, H. and Mizushima, S. (1991) Proc. Natl. Acad. Sci. USA, 88, 6545-6549.
2) Matsuyama, S., Fujita, Y., Sagara, K. and Mizushima, S. (1992) Biochim. Biophys. Acta, 1122, 72-84.

H 129 LEADER PEPTIDASE BELONGS TO A NEW CLASS OF SERINE PROTEASES, NOT REQUIRING A HISTIDINE RESIDUE AS A GENERAL BASE, Meesook Sung and Ross E. Dalbey, Department of Chemistry, The Ohio State University, Columbus, OH 43210

Leader peptidase of Escherichia coli cleaves the leader sequence from the amino terminus of membrane and secreted proteins after these proteins insert across the membrane. Despite considerable research, the mechanism of catalysis of leader peptidase remains unknown. This peptidase cannot be classified using protease inhibitors to the serine, cysteine, aspartic acid, or metallo classes of proteases. Using site-directed mutagenesis, we have attempted to place leader peptidase in one of these groups. We found that leader peptidase, lacking all of the cysteine residues, can cleave the leader peptide from procoat, the precursor to bacteriophage M13 coat protein. Substitution of each histidine residue with an alanyl residue was without effect on catalysis. In contrast, the substitution of serine 90 and serine 185 as well as aspartic acid 99, 153, 273, and 276 with an alanyl residue strongly inhibits the processing of procoat. However, only serine 90 and aspartic acid 153 were required for cleavage using a highly sensitive in vivo assay. The serine 90 is conserved in all known prokaryotic and eukaryotic signal peptidases. In addition, catalysis can still occur in leader peptidase, when serine 90 is changed to a cysteine. In contrast to the wild-type enzyme, this mutant leader peptidase is inactivated by N-ethylmaleimide, a cysteine-specific reagent. This study thus defines two critical residues, serine 90 and aspartic acid 153, that may be directly involved in catalysis and provides strong evidence that leader peptidase belongs to a novel class of serine proteases, not requiring a histidine residue.

H 130 IDENTIFICATION OF A PUTATIVE RECEPTOR REQUIRED FOR PROTEIN ACCUMULATION WITHIN THE ER LUMEN OF PLANTS, Jürgen Denecke, Lena E. Carlsson, Sabina Vidal, Bo Ek* and E. Tapio Palva, Dept. Molecular Genetics, *Dept. Cell Research, Swedish University of Agricultural Sciences, Uppsala, Sweden

We analyzed the requirements for retention of proteins in the lumen of the endoplasmic reticulum (ER) of tobacco protoplasts using the enzyme phosphinothricin acetyl transferase (PAT) as a heterologous passenger protein. The transport experiments were conducted with the help of a transient expression system using PAT derivatives carrying C-terminally added sequences. This work allowed us to conclude that ER localization signals are not sufficiently described by the sequence comprising the last 4 aminoacids of a protein, but that such sorting signals should be classified in terms of structures (Denecke et al., 1992a). We could demonstrate that ER localization signals are structurally conserved between mammals and plants but not the yeast S. cerevisiae. Three major reactive plant proteins (RPL 60, RPL 75 and RPL 90) were purified based on their binding to a monoclonal antibody that recognizes this structure. We show that these proteins are similar to calreticulin (CRP55), the luminal binding protein (BiP/GRP78/KAR2) and endoplasmin (ERP99/GRP94). The corresponding anti-idiotype antibodies are currently used to identify putative salvage receptors in plants. A candidate protein of 40 kDa has been identified and experiments are done to localize this protein within a specific compartment of the plant endomembrane system. A possible strategy for the selection and isolation of plant mutants with a modified retention specificity, based on the use of PAT as a selectable reporter, is presented as well.

Denecke, J., De Rycke, R. and Botterman, J. (1992). Plant- and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. EMBO J., 6, 2345-2355.

H 131 MULTIPLE PATHWAYS FOR PROTEIN TRANSLOCATION INTO THYLAKOID MEMBRANES. Kenneth Cline, Ralph Henry, Chang-Jiang Li, and Jianguo Yuan. Horticultural Sciences Department, University of Florida, Gainesville, FL 32611.

Nuclear-encoded and cytosolically-synthesized thylakoid proteins are localized via sequential transmembrane translocation processes. A chloroplast envelope-based system delivers the proteins to the aqueous stromal matrix. A thylakoid-based translocation system transports the stromal intermediates into or across the thylakoid bilayer. Cline et al (J Biol Chem 267:2688-2696, 1992) recently showed that the energetics for thylakoid translocation is protein specific and ranges from total dependence on ATP to the combined use of ATP and a proton gradient to the total dependence on a proton gradient. This suggested the possibility of multiple thylakoid translocators. To test this possibility, precursors prepared by overexpression in *E. coli* were used to saturate the translocators and compete for translocation with radiolabeled precursors. Saturation of thylakoid translocators occurred *in organello* during import reactions because import into the chloroplast occurred much more rapidly than subsequent translocation into thylakoids. Competition under these conditions revealed 3 specificity groups: OE23 and OE17; OE33 and plastocyanin; and LHCP. Using assays for translocation into isolated thylakoids it was possible to show that OE23 competition occurs on membrane translocation sites, demonstrating that OE23 and OE17 utilize a different translocase than the other proteins. LHCP integration and OE33 transport depend upon soluble factors; current experiments are designed to determine whether saturation and competition with these proteins is occurring at the level of soluble factors or the membrane translocase. Precursor specificity of the various translocators will be discussed within the context of the endosymbiotic origins of chloroplasts.

H 132 CHEMICAL INHIBITORS OF THE CHLOROPLAST PROTEIN TRANSLOCATION APPARATUS, Sidney Varian Scott and Steven M. Theg, Section of Botany, University of California-Davis, Davis, CA 95616.

To address the question of how nuclear-encoded proteins are targeted to organelles, the action of a number of drugs which effect protein import into chloroplasts was investigated. Two diverse classes of chemicals, L-type calcium channel blockers and cytotoxic drugs used in cancer therapy, were found to inhibit the membrane translocation step of protein import into pea chloroplasts. Nifedipine, a well-characterized calcium channel blocker, does not inhibit the specific binding of the precursor protein to the chloroplast outer envelope, but rather blocks the membrane translocation step of the protein import reaction. Experiments in which Ca^{2+} was either added exogenously or chelated with EDTA showed that the inhibitory effect of calcium channel blockers on protein import is not solely a result of altered Ca^{2+} levels within the chloroplast. These experiments suggest that the chloroplast polypeptide targeted by the chemical inhibitors does not function as a Ca^{2+} channel, but rather may mediate the transport of proteins across chloroplast envelope membranes.

Although the chemicals used to inhibit chloroplast protein import are structurally and functionally diverse, they are known to be transported out of mammalian cells that overexpress P-glycoprotein, the ATP-dependent drug pump implicated in multidrug resistance. P-glycoprotein is a member of a superfamily of transport proteins which includes, among others, the cystic fibrosis transmembrane conductance regulator, the hemolysin and maltose translocators in *E.coli*, and the *ste6* gene product in yeast. Immunoblots of chloroplast outer envelope membranes react with three P-glycoprotein-specific antibodies; a monoclonal antibody raised to hamster P-glycoprotein, and two polyclonal antibodies raised to synthetic peptides derived from human P-glycoprotein. These experiments are consistent with a working model which postulates that the chloroplastic immunoreactive protein is an ATP-requiring translocator of the P-glycoprotein superfamily, and that the chemical inhibitors described above act by competing with chloroplast precursor proteins for binding sites on this polypeptide.

H 133 IMPORT OF PROTEINS INTO CHLOROPLASTS CAN PROCEED AT SURPRISINGLY LOW TEMPERATURES,

Steven M. Theg and Ellen A. Leheny, Section of Botany, University of California-Davis, Davis, CA 95616

With the exception of a small number of proteins in chloroplasts and mitochondria, the proteins present in the organelles of eukaryotic cells are made from nuclear-encoded genes and are taken up either during or after their synthesis on cytoplasmic ribosomes. This uptake process necessarily involves the translocation of proteins across a organellar membranes. In virtually all membranes engaged in protein translocation, the transport step itself has been reported to be inhibited by low temperatures.

In isolated chloroplasts, the temperature dependence of protein translocation across the envelope membranes is particularly steep. Reactions that occur with high rates at 25°C proceed many times more slowly at 10°C and essentially not at all at 4°C. It is paradoxical, then, that some plants are able to adapt to growth at temperatures as low as or colder than 4°C. In an attempt to understand this phenomenon, we compared the temperature profile for protein import into chloroplasts isolated from peas grown at 4-7°C and at 25°C. Surprisingly, we found that this temperature profile was indistinguishable for the two chloroplast types. Furthermore, while neither chloroplast type was able to import proteins at 4°C when kept in the dark, they were both able to do so when illuminated. Investigation of this phenomenon revealed that the effect of illumination was to allow ATP to be synthesized in the stroma, the only location from which its hydrolysis can drive the protein import reaction. Guided by this discovery, we traced the well-documented inhibition of chloroplast protein uptake by low temperatures in the dark to an inability of the adenylate translocator to transport exogenously added ATP to the stroma at these low temperatures. These experiments demonstrate that the cold-induced inhibition of protein translocation across membranes may not be related to the formation of nonpermissive lipid fluidity states at low temperatures.

H 134 SIGNAL SEQUENCE MUTATIONS AND THEIR SUPPRESSORS INVOLVED IN THYLAKOID MEMBRANE PROTEIN TRANSLOCATION,

Tracy A. Smith, Audrey L. Alexander, and Bruce D. Kohorn, Botany Department, Duke University, Durham, NC 27708

We are examining the translocation of proteins across the thylakoid membrane of chloroplasts, a process about which little is known. Cytochrome f, which is required for photosynthesis, is a chloroplast encoded protein that spans the thylakoid membrane once. Cyt f has a 30 amino acid N-terminal cleaved sequence that has homology to bacterial signal sequences. We made changes in the putative signal sequence by site directed mutagenesis and introduced the altered protein into the chloroplasts of the eukaryotic algae *Chlamydomonas* by transformation and homologous recombination. The introduction of single positive charges into the hydrophobic core domain of the putative signal sequence causes the loss of cyt f as detected by Western blot and prohibits these transformants from growing photosynthetically. Additional signal peptide mutations are being studied. We are attempting to isolate extragenic suppressors of these mutations by selecting for the restored ability of cells to grow photosynthetically. These extragenic suppressors should identify components of the insertion machinery that interact with cyt f in chloroplasts. Similar studies are being carried out on a subunit of the thylakoid ATPase.

H 135 PREFERREDOXIN IMPORT INTO CHLOROPLASTS

Marinus Pilon, Twan America, Ron van 't Hof, Peter Weisbeek and Ben de Kruijff.
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Chloroplasts depend largely on cytosolic protein synthesis. An envelope, consisting of two membranes separates the organel from the cytosol. In order to be imported and correctly routed precursor proteins contain topogenic information in a cleavable N-terminal extension, called the transit sequence. The import pathway of the stromal protein ferredoxin was investigated in detail with the aid of a purified precursor. Our analysis revealed that the precursor of ferredoxin (prefd) is translocation competent by itself, indicating that recognition of the topogenic information takes place at the envelope and is not mediated by cytosolic factors. Import is ATP-dependent and follows saturation kinetics. In order to perform a structural analysis of prefd and in order to localize structural elements in its functional domains the full precursor, the transit peptide and the mature protein, both in holo- and apo-form were obtained and analyzed separately. Circular Dichroism, tryptophan fluorescence quenching, protease accessibility and gelfiltration indicated that prefd is largely unstructured and resembles unfolded proteins. These properties are due to both mature part and transit sequence. The high conformational flexibility of the transit sequence, and its lipid-penetrating capacity is of possible importance to the import process.

At present we are using two approaches to gain more insight into the mechanism of chloroplast targeting. Firstly, in an attempt to link functional properties of the transit sequence to structural features we started with the analysis of a specific set of mutations in the transit sequence. Secondly, a cross-linking approach is used to tag components of the translocation machinary that interact with the precursor during envelope transit. Recent results will be presented on the poster.

H 136 POST-TRANSLATIONAL INSERTION OF A THYLAKOID MEMBRANE PROTEIN.

Bruce D. Kohorn, Botany Dept. Duke University, Durham, NC 27706.

Many of the proteins integral to the chloroplast thylakoid membrane must be synthesized in the cytoplasm, and so their site of synthesis has thought to be distinct in time and space from their membrane integration. The Light Harvesting Chlorophyll a/b binding protein (LHCP) is synthesized as a cytoplasmic precursor and can be imported post-translationally into isolated chloroplasts to form a soluble complex with a stromal factor (Payan and Cline 1991, JCB 112, 603). .The formation of the LHCP-stromal complex is required for subsequent LHCP insertion into isolated thylakoids. Unlike many membrane proteins, the integration of LHCP into the thylakoid requires three hydrophobic α-helices of the mature protein, and seems not to be initiated by a short signal sequence (Auchincloss *et al* 1992, JBC 267, 10439). To identify proteins required for LHCP translocation, I generated an antidiotypic antibody to mature LHCP (termed ααL). ααL serum, but not preimmune, specifically reacts with a 60 kDa soluble stromal protein. Fractionation of native stroma on Sephacryl S300 shows that the 60 kDa protein elutes with the stromal activity required for LHCP insertion. Further characterization using isolated thylakoids and stroma should reveal the role that this 60 kDa protein plays in thylakoid protein integration.

H 137 THE ROLE OF PROTEIN CONFORMATION ON THE IMPORT COMPETENCE OF THE OVER-EXPRESSED PRECURSOR, prSSU. Melissa Hurley, Barry D. Bruce, & Kenneth Keegstra, Department of Botany, University of Wisconsin at Madison, Madison, WI. USA 53706

Both the precursor and mature forms of the small subunit of tobacco Rubisco (SSU) have been over-expressed in *E. coli* and purified to homogeneity. The precursor is competent for import into isolated pea chloroplasts and for processing into its mature form. This import is ATP-dependent but does not require the addition of any cytosolic factors. However, this import competence is labile and is lost as a function of time after dilution from 8M urea or 6M guanidine-HCl. We are investigating the accompanying conformational changes of this protein using traditional structural techniques including tryptophan fluorescence, limited proteolysis, and size-exclusion HPLC. All three of these techniques indicate that the loss of import competence of prSSU correlates with a transition of prSSU from an unfolded and/or aggregated form into a more compact, folded conformation. Import competence is maintained with time under conditions which slow or alter folding; at low temperatures or in the presence of DTT. The percent of import competent molecules can also be increased by co-incubation with a purified plant cytosolic hsp70, a putative molecular chaperone for this system. These results are consistent with the hypothesis that protein translocation across the chloroplast envelope requires an unfolded precursor.

H 138 THE ISOLATION OF VARIOUS HIGHER PLANT PLASTID SUBTYPES BY MAGNETIC IMMUNO-ABSORPTION. Barry D. Bruce[1] & Albert P. Kausch[2], Botany Dept.[1], University of Wisconsin, Madison, WI 53706 & DeKalb Plant Genetics[2], Eastern Point Rd., Groton, CT 06340 USA

Antibodies have been prepared which are specific to the outer membrane of the chloroplast envelope. This antisera is immuno-reactive with a variety of plastid forms from both monocotyledonous and dicotyledonous plants. By coupling this antibody to magnetic nanoparticles we have rapidly and gently isolated physiologically intact organelles on a steel wool matrix suspended in a 0.6 Tesla magnetic field. Using this magnetic immunoabsorption procedure we have been able to isolate pure chromoplasts, amyloplasts, and chloroplasts from whole cell lysates of several plant species. The integrity of these plastids has been demonstrated by *in organellar* protein synthesis, ^{14}C-ADP-glucose uptake, flow cytometry, *in vitro* synthesized precursor import and FITC-cationized ferritin staining of the plastid envelope. The ability to isolate intact nongreen plastids will provide a superior physiological system for the study of many plant processes. Magnetic immuonabsorption is a new method that may be useful in the *in vitro* analysis of many different cellular compartments from a wide range of organisms.

H 139 Analysis of peroxisomal protein import in a permeabilized cell system, Martin Wendland and Suresh Subramani, Department of Biology, University of California, San Diego, La Jolla, California 92093
Using streptolysin-O (SLO) we have developed a permeabilized cell system retaining the competence to import proteins into peroxisomes. We used luciferase and albumin conjugated with a peptide ending in the peroxisomal targeting signal, SKL, to monitor the import of proteins into peroxisomes. After incubation with SLO-permeabilized cells, these exogenous proteins accumulated within catalase-containing vesicles. The import was strictly signal dependent and could be blocked by a 10-fold excess of a peptide containing the SKL-targeting signal, while a control peptide did not affect the import. Peroxisomal accumulation of proteins was time and temperature dependent and required ATP-hydrolysis. Dissipation of the membrane potential did not alter the import efficiency. GTP-hydrolysing proteins were not required for peroxisomal protein targeting. Depletion of endogenous cytosol from permeabilized cells abolished the competence to import proteins into peroxisomes but import was reconstituted by the addition of external cytosol. We present evidence that cytosol contains factors with SKL-specific binding sites. The activity of cytosol is insensitive to N-ethylmaleimide (NEM) treatment, while the cells contain NEM-sensitive membrane-bound or associated proteins which are involved in the import machinery. The cytosol dependence and NEM-sensitivity of peroxisomal protein import should facilitate the purification of proteins involved in the import of proteins into peroxisomes.

H 140 A PEROXISOMAL ASSEMBLY MUTANT IN THE YEAST *PICHIA PASTORIS*, Allan P. Spong and Suresh Subramani, Dept. of Biology, 0322, UCSD, La Jolla, CA 92093.
The establishment of a genetic system in the methylotrophic yeast *Pichia pastoris* to study peroxisomal biogenesis has yielded several different complementation groups of peroxisome assembly (*pas*) mutants. Each appears to be deficient for functional peroxisomes. One of these, *pas5*, has been characterized, complemented and sequenced. Ultrastructural studies show that normal peroxisomes are not present in *pas5*, but aberrant peroxisomal structures are frequently observed. These structures appear to be induced, and segregated to daughter cells normally. These structures may be related to the peroxisomal ghosts reported in the human condition Zellweger syndrome.
Fractionation analysis of peroxisomes from the *pas5* mutant reveals that many peroxisomal enzymes are induced normally but are not targeted to the peroxisome. Import of proteins with either carboxy- or amino- terminal targeting signals are affected. The *P. pastoris PAS5* encodes a 127KDa protein, which contains a 200 amino acid long region of homology with the *S. cerevisiae* PAS1 protein. This same region is homologous to NSF and other related ATPases. Progress on the role of this protein in peroxisome assembly will be described.

H 141 IMPORT OF A MITOCHONDRIAL PRESEQUENCE INTO PROTEIN-FREE PHOSPHOLIPID VESICLES, David Roise and Merritt Maduke, Department of Chemistry, University of California, San Diego, La Jolla, CA. 92093-0506

A synthetic mitochondrial presequence has been shown to translocate across pure phospholipid bilayers. The presequence corresponds to the amino-terminal 25 residues of the precursor form of yeast cytochrome oxidase subunit IV. It was fluorescently labeled so that its association with large unilamellar vesicles could be monitored by steady state fluorescence spectroscopy. When bound to vesicles across which a transmembrane electrical potential with negative internal polarity had been imposed, the fluorescent presequence showed time-dependent protection both from proteolytic digestion by trypsin and from chemical reduction of the fluorescent label by sodium dithionite. The protected presequence remained associated with the vesicles upon gel filtration but could be released by treatment of the vesicles with detergent. The kinetics of protection from both trypsin and dithionite were similar, and both of the rates displayed a combination of first-order and second-order dependence on the concentration of bound presequence. The results show that a mitochondrial presequence can be translocated into phospholipid vesicles in a potential-dependent manner. The specific targeting of precursor proteins to mitochondria *in vivo* may depend on this behavior.

H 142 **PROTEIN TRANSPORT TO HIGHER PLANT PEROXISOMES** **Laura J. Olsen, William F. Ettinger and John J. Harada,** Division of Biological Sciences, Section of Botany, University of California, Davis, CA 95616.

There are at least three defined types of higher plant peroxisomes that differ in metabolic function and tissue localization. Glyoxysomes are found primarily in germinating seedlings and are involved in mobilizing fatty acids via the glyoxylate cycle and beta-oxidation pathway. Glyoxysomes have also been reported in senescing leaves and pollen. During greening of seedlings, glyoxysomes become leaf peroxisomes, specialized for photorespiration. In root nodules of some plants, root peroxisomes contain enzymes for nitrogen metabolism. The biogenesis of these organelles necessarily requires the transport of proteins from their site of synthesis in the cytoplasm to their final location in the peroxisome matrix or membrane. We are studying the developmental specificity of this process as well as the targeting determinants involved. *Arabidopsis thaliana* plants transformed with a full-length cDNA for isocitrate lyase (a key enzyme in the glyoxylate cycle) under the control of a constitutive promoter were analyzed to see if this glyoxysomal protein was imported into leaf peroxisomes. We found that leaf peroxisomes were competent to transport the glyoxysomal protein isocitrate lyase. The deletion of 37 amino acids from the carboxyl terminus of isocitrate lyase resulted in the localization of this deletion mutant to the cytosol of transgenic plants. As few as 4 amino acids from the extreme carboxyl terminus of isocitrate lyase attached to a passenger protein were sufficient to target the passenger protein to leaf peroxisomes. Therefore, the carboxyl terminus of isocitrate lyase is both necessary and sufficient to target proteins to peroxisomes. We have recently begun to develop an *in vitro* assay to study the mechanism(s) of import of proteins into glyoxysomes. Preliminary results with this assay indicate that the process is time-, temperature- and energy-dependent. An *in vitro* assay will be a powerful tool to identify components of the import apparatus and to further study protein transport into peroxisomes.

H 143 PEROXISOME BIOGENESIS IN THE METHYLOTROPHIC YEAST *HANSENULA POLYMORPHA*: GENETIC ANALYSIS OF HEAT SHOCK PROTEINS (HSP70), Rainer Roggenkamp and André Diesel, Institute of Microbiology, University of Düsseldorf, FRG.

Upon methylotrophic growth large and numerous peroxisomes are induced in *H. polymorpha*. The abundant peroxisomal proteins MOX, DAS and catalase are imported posttranslationally via particular tripeptides at their C-termini. Assembly of these proteins occurs in a regulated fashion; MOX forms a crystalloid core and the DAS molecules are found within this crystalloid structure. In contrast, catalase is located in the matrix space between the core and the peroxisomal membrane. In order to demonstrate a possible role of molecular chaperones facilitating import and assembly we have started to analyse genes coding for proteins of the HSP70 family. Polymerase chain reaction using synthetic oligonucleotides homologous to conserved regions in HSP70 genes was performed in order to obtain a fragment of 1.5 kb in length. Southern analysis using this fragment as a probe revealed that the gene family coding for HSP70 proteins in *H. polymorpha* comprises approximately half of the number of genes as compared to *S. cerevisiae*. The 1.5 kb fragment was also used for an initial screening of a genomic library of *H. polymorpha*. Two different genes could be isolated by colony hybridization. Upon partial sequencing one gene showed the highest homology to the *SSA* genes of *S.cerevisiae*, the other to the mitochondrial *SSC* gene product of *S. cerevisiae*. Further sequence analyses and gene disruption experiments are currently performed in order to test the genes for a possible function in peroxisomal protein transport.

H 144 **THE *PICHIA PASTORIS* PAS4 GENE REQUIRED FOR PEROXISOME ASSEMBLY ENCODES A UBIQUITIN CONJUGATING ENZYME,** Stephen J. Gould and Dennis I. Crane, Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine and Kennedy Krieger Research Institute, Baltimore, MD 21205.

The peroxisome is a distinct organelle present in almost all eukaryotic cells and is the site of diverse metabolic activities. Though this organelle does not appear essential for cell viability, defects in the assembly of the peroxisome cause several lethal genetic disorders in humans. In an effort to characterize the genes, gene products, and mechanisms involved in peroxisome assembly, we have initiated a genetic analysis of this process in yeast. We report here the cloning and initial characterization of PAS4, a gene required for peroxisome assembly in the yeast Pichia pastoris. Comparison of the deduced amino acid sequence of PAS4 with known protein sequences reveals that it has a high degree of similarity to several genes that encode ubiquitin-conjugating enzymes (RAD6, UBC1, CDC34). Based upon the sequence similarity between the PAS4 protein (Pas4p) and ubiquitin conjugating enzymes, the ability of Pas4p to form thiolester conjugates with ubiquitin, and the inactivation of PAS4 by mutations that replace the putative active site cysteine residue with alanine or serine, we conclude that PAS4 is a member of the ubiquitin-conjugating enzyme gene family. Studies on the subcellular distribution of this protein will also be presented. These findings suggest that protein ubiquitination plays an essential role in the assembly of peroxisomes.

H 145 THE *PAS8* GENE OF *PICHIA PASTORIS* IS ESSENTIAL FOR IMPORT OF PEROXISOMAL PROTEINS CONTAINING THE PTS-1,BUT NOT PTS-2, TARGETING SIGNAL, D. McCollum, E. Monosov, and S. Subramani, Dept. of Biology, 0322, UCSD, La Jolla, CA 92093.
We have isolated 8 complementation groups of peroxisome assembly (*pas*) mutants of the yeast *Pichia pastoris*. Here we describe the characterization of one of these mutants, *pas8*, and the cloning of the *PAS8* gene. The *pas8* mutant is deficient for growth, but not for division of peroxisomes or induction of peroxisomal proteins. Two distinct peroxisomal targeting signals, PTS-1 and PTS-2, have been identified that are sufficient to direct proteins to the peroxisomal matrix. In this report we show that the *pas8* mutant is deficient in the import of proteins with the PTS-1, but not the PTS-2, targeting signal. This is the same import deficiency as is found in cells from patients with the lethal human peroxisomal disorder Zellweger Syndrome. Furthermore, all other *pas* mutants of *P. pastoris* appear to be unable to import both PTS-1- and PTS-2-containing proteins, suggesting that PAS8 may be the PTS-1 recepter. We also report the cloning and sequence of the *PAS8* gene, which encodes a novel member of the tricotetrapeptide repeat gene family, and the localization of the PAS8 gene product.

H 146 *ALTERNATIVE TOPOGENIC SIGNALS OF PEROXISOMAL AND MITOCHONDRIAL CITRATE SYNTHASE IN YEAST*, Alfred S. Lewin[1], Keshav K. Singh[1], Gillian M. Small[2], [1]Department Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, FL 32610, and [2]Department of Cell Biology and Anatomy, Mt. Sinai Medical Center, New York, NY 10029
The tripeptide serine-lysine-leucine (SKL) occurs at the carboxyl terminus of many peroxisomal proteins and serves as a peroxisomal targeting signal. *Saccharomyces cerevisiae* has two isozymes of citrate synthase. The peroxisomal form, encoded by *CIT2*, terminates in SKL, while the mitochondrial form, encoded by *CIT1*, begins with an amino terminal mitochondrial signal sequence and ends in SKN. We analyzed the importance of SKL as a topogenic signal for citrate synthase, using oleate to induce peroxisomes and density gradients to fractionate organelles. These results were substantiated using immunocytology. Our experiments revealed that SKL was necessary for directing citrate synthase (cit2) to peroxisomes, and that the amino terminal leader was required for targeting cit1 protein to mitochondria. C-terminal SKL was also sufficient to target a leaderless version of mitochondrial citrate synthase to peroxisomes. Deleting this tripeptide from the cit2 protein caused peroxisomal citrate synthase to be missorted to mitochondria. When the first 18 amino acids of this version of cit2 protein (cit2-SKL) were also removed by genetic deletion, then the protein remained in the cytosolic fraction. These experiments suggest that the cit2 protein contains a cryptic mitochondrial targeting signal. In the mitochondrion, cit2 protein was not functionally identical to cit1 protein: cells containing mitochondrial cit2 were not able to grow on acetate medium. Cit1 protein could support acetate growth when present either in the mitochondria or the cytoplasm, but not when targeted to peroxisomes. This result suggests a siphoning of reactants toward the non-energy producing oxidations of the peroxisome when cit1 is mislocalized.

H 147 *MAS6* ENCODES AN ESSENTIAL INNER MEMBRANE PROTEIN REQUIRED FOR MITOCHONDRIAL PROTEIN IMPORT. Robert E. Jensen, Jennifer L. T. Emtage, Kathleen Ryan, and Jennifer Kalish, Dept. of Cell Biology and Anatomy, Johns Hopkins School of Medicine., Baltimore, MD 21205

Mitochondrial protein import is a multistep process that includes binding of a precursor protein to outer membrane receptors, translocation of the polypeptide across one or both mitochondrial membranes, proteolytic cleavage of the presequence, and refolding of the imported protein to an active conformation. To identify the components that mediate protein import into mitochondria, we are analyzing yeast mutants defective in import. *mas6* mutants are temperature-sensitive for viability, and accumulate mitochondrial precursor proteins at the non-permissive temperature. *MAS6* encodes a 23 kDa protein that contains 3 or 4 potential membrane spanning domains, and yeast strains disrupted for *MAS6* are inviable at all temperatures and on all carbon sources. The MAS6 protein is located in the mitochondrial inner membrane and cannot be extracted from the membrane by high salt or alkali treatment. Antibodies to the MAS6 protein inhibit import into isolated mitochondria, but only when the outer membrane has been disrupted by osmotic shock. *MAS6* therefore represents the first essential inner membrane protein import component.

Mitochondria isolated from strains that carry the *mas6-1* mutation are defective at an early step in the translocation of precursor proteins across the mitochondrial membranes. In particular, very little precursor pellets with the *mas6-1* mitochondria after the import reaction. In timecourse studies, the precursor appears to initially bind to the mitochondrial surface, but then is released from the mitochondria at a later import step. To determine the precise role of MAS6 in import, we are isolating additional *mas6* alleles, and analyzing their effects on import.

The MAS6 protein does not contain a amino-terminal, cleavable presequence typical of most imported proteins. To identify the signal(s) that targets MAS6 to the inner membrane, we have constructed a series of truncated MAS6 proteins, and are currently testing which of these altered MAS6 proteins are imported into mitochondria.

H 148 MEMBRANE TOPOLOGY OF THE GASTRIC H,K-ATPase α-SUBUNIT. Krister Bamberg and George Sachs, UCLA and VA Wadsworth, B.113, Rm.324, Los Angeles, CA 90073.
The functional gastric H,K-ATPase is a membrane protein that consists of at least two subunits, a catalytic α-subunit (1035 amino acids) and a glycosylated ß-subunit (291 amino acids) of unknown function. We are interested in the membrane topology of the α-subunit and present a convenient system that can be used readily to define putative transmembrane segments.

A cDNA encoding a fusion protein of the 102 most N-terminal amino acids of the rabbit H,K-ATPase α-subunit linked by a variable segment to the 177 most C-terminal amino acids of the rabbit H,K-ATPase ß-subunit is expressed in a rabbit reticulocyte lysate system in the absence or presence of dog pancreatic microsomes. Because the fusion protein retains 5 of the originally 7 consensus N-glycosylation sites present in the H,K-ATPase ß-subunit, translocation of the C-terminal ß-subunit part of the fusion protein into the microsomes can be evidenced by glycosylation. Glycosylation of the fusion protein is therefore evidence for an odd number of transmembrane segments in the variable region preceding the ß-subunit part. The variable region is synthesized using PCR on any region of interest within the HK-ATPase α-subunit cDNA and ligated into the construct. Radioactive translation products from reactions with and without microsomes are then separated on SDS polyacrylamide gels and subjected to autoradiography. A glycosylated fusion protein has greatly reduced mobility in the gel when compared to its non-glycosylated counterpart obtained in the absence of microsomes.

The validity of the system is demonstrated using four constructs termed M0, M1, M2 and M12. The variable segment in M0 is a stretch of six nonhydrophobic aminoacids, whereas in M1 and M2 it is the first and second hydrophobic segment of the H,K-ATPase α-subunit, respectively. M12 contains the first two hydrophobic segments. These regions of the H,K-ATPase α-subunit were choosen as controls because M1 and M2 have been established by other methods to be transmembraneous (Munson et al. 1991; JBC 266, 18976-88). M0 and M12, with none or two transmembrane segments in the variable region, respectively, were translated into non-glycosylated fusion proteins. M1 and M2, with one transmembrane segment in the variable part, were translated into glycosylated fusion proteins. We therefore conclude that our system performs as expected and is usefull to determine whether potential transmembrane segments of the H,K-ATPase α-subunit actually do span the membrane. Evaluation of additional constructs should lead to a more complete picture of the transmembraneous organization of the H,K-ATPase α-subunit.

H 149 INHIBITION OF THE PEROXISOMAL IMPORT OF A MICRO-INJECTED PROTEIN BY COINJECTION OF ANTIBODIES TO MEMBERS OF THE 70 kD HEAT SHOCK PROTEIN FAMILY.
Paul A. Walton*, Jean Pierre Morello*, and William J. Welch†. *Dept. of Anatomy and Cell Biology, McGill University Montreal, Canada; and †Dept. Medicine and Physiology, University of California-San Francisco.

Proteins destined for the peroxisomal matrix are synthesized on free polysomes in the cytoplasm and imported by a post-translational mechanism. Microinjection of a hybrid protein containing a peroxisomal targeting signal (HSA-SKL) has been demonstrated to yield an *in vivo* assay resulting in the import of that protein into the peroxisomal matrix (Walton, et al. 1992 *Molec.Cell.Biol.*12,531-541). In order to determine if heat shock proteins of the 70 kD family (HSP70) play a role in the import of proteins into the peroxisomal matrix, a mixture of monoclonal antibodies directed against constitutive (73kD) and inducible (72kD) HSP70 were coinjected with HSA-SKL into human fibroblasts. This mixture of antibodies has previously been shown to inhibit the heat shock response of mammalian fibroblasts (Riabowol, et al. 1988 *Science* 242,433-436). The import of the hybrid protein was inhibited by coinjection of the mixture of monoclonal antibodies, but only in the presence of cycloheximide. Cycloheximide was included to inhibit the synthesis of the inducible form of the HSP70 which occurs following microinjection of anti-HSP70 antibodies. Import was uninhibited by cycloheximide alone or by the microinjection of the mixed monoclonal antibodies in the absence of cycloheximide. In addition, import was not inhibited by coinjection of monoclonal antibodies or affinity-purified polyclonal antibodies directed against the inducible form of HSP70, even in the presence of cycloheximide. The presence of the microinjected hybrid protein elicited a heat shock response in these cells approximately 8 hours after microinjection as judged by immuno-cytochemistry for the expression of the inducible HSP70 proteins.

These results suggest that a constitutive member of the HSP70 family is involved in the import of proteins into the peroxisomal matrix, and that proteins of the inducible HSP70 family can also act in this regard.

H 150 Surf 4: A Conserved Membrane Protein Found Exclusively in Vesicles,
Julie E. Reeves and Mike Fried, Eukaryotic Gene Organization and Expression, Imperial Cencer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London, UK WC2A 3PX.

The predicted amino acid sequence of mouse Surf 4 gene (Huxley and Fried, MCB 1990) has seven potential transmembrane domains oriented with the carboxy terminus in the cytoplasm. This carboxy terminus contains a double lysine motif for retention to the ER. To determine Surf 4's cellular location, a c-myc tag was inserted into the Surf 4 cDNA and stable transfectants expressing the c-myc tagged protein were made. In order to study endogenous Surf 4 protein, mice and rabbits were immunised with peptides based on the predicted Surf 4 amino acid sequence.
Immunofluorescence with either mAB 9E10, specific for the c-myc tag, or antisera raised against Surf 4 peptides detects cytoplasmic vesicles. Double labelling experiments suggest that Surf 4 protein does not colocalise with marker antibodies for the intermediate compartment, lysosomes, endosomes or mitchondria. Surf 4 may be a marker for a new compartment, this is being investigated further at present. In addition, Surf 4 appears to be conserved from humans to nematodes in terms of structure and cellular location, based on DNA sequence analysis and immunofluorescence staining.

H 151 *Abstract Withdrawn*

Membrane Traffic in Mammalian Cells

H 201 RETENTION OF THE HIV ENVELOPE GLYCOPROTEINS IN THE ENDOPLASMIC RETICULUM BY CD4 MOLECULES BEARING THE TRANSMEMBRANE ER RETENTION SIGNAL: ANALYSIS OF THE ENDOPROTEOLYTIC CLEAVAGE OF GP160, M. Abdul Jabbar, Nicholas U. Raja and Martin J. Vincent, Department of Molecular Biology, Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195.

We have investigated endoproteolytic processing of the HIV envelope glycoprotein precursor, gp160, as well as envelope-mediated membrane fusion in the presence of CD4 molecules that were either partially or fully retained in the endoplasmic reticulum (ER). The genes encoding gp160 and CD4 molecules were introduced into mammalian cells and expression analyzed using the vaccinia virus-T7 RNA polymerase transient expression system. Pulse-chase analysis revealed that gp160 formed complexes with the CD4 molecules, and gp160 in the complex was endoproteolytically cleaved to gp120 and gp41 in the intracellular compartment. The gp120/gp41 complex thus generated was properly targeted to the plasma membrane in cells expressing gp160 and wtCD4 or mutant CD4 molecules that were partially retained in the ER. However, the intracellular gp120/gp41 complex did not reach the plasma membrane in cells expressing gp160 and mutant CD4 that was fully retained in the ER, as evidenced by the lack of gp120 shedding into the medium. Furthermore, we performed membrane fusion assay to monitor the presence/absence of the gp120/gp41 complex at the cell surface of cotransfected cells and demonstrated that the HIV envelope-mediated membrane fusion could not occur in the presence of wtCD4 or either one of the CD4 molecules bearing the transmembrane ER retention signal. Thus these data suggested that the endoproteolytic cleavage of gp160 had occurred in a membrane compartment topologically proximal to the ER, perhaps the cis-Golgi network, and the retention approach could have a general application in the analysis of complex viral and cellular glycoprotein synthesis and function.

H 202 TARGETING OF ERGIC-53 TO THE ER-GOLGI INTERMEDIATE COMPARTMENT, Christian Itin, Richard Schindler, Felix Kappeler and Hans-Peter Hauri, Department of Pharmacology, Biocenter of the University of Basel, CH-4056 Basel, Switzerland

ERGIC-53 is a marker protein for the ER-Golgi intermediate compartment (ERGIC). Cloning of the cDNA of ERGIC-53 revealed a transmembrane protein with type I topology. Surprizingly the 12 amino acid long cytoplasmic tail carries a KKXX ER retention motif although ERGIC-53 localizes to a post-ER compartment. This raised the question whether the KKXX is a functional motif. Therefore we replaced the cytoplasmic tail of a surface membrane protein (human CD4) with that of ERGIC-53. The construct was efficiently retained when transiently expressed in Vero and COS cells showing an ER-like pattern by immunofluorescence microscopy clearly different from endogenous ERGIC-53. Replacement of the two lysines by serines in this construct lead to surface appearance that was indistinguishable from expressed human CD4. This indicates that the KKXX-motif in the cytoplasmic tail of ERGIC-53 acts as a retention signal without conferring ERGIC localization. In contrast, overexpression of ERGIC-53 in Vero and COS cells lead to surface appearance, whereas low levels of expression in Vero cells revealed correct intracellular localization to the ERGIC suggesting saturation of ERGIC-53 retention. To test if the transmembrane domain of ERGIC-53 affects retention we attached the transmembrane and the cytoplasmic domain of ERGIC-53 to the luminal portion of CD4. This construct was efficiently retained in the ER without missorting to the plasma membrane indicating that the transmembrane together with the cytoplasmic domain is not sufficient for correct targeting to the ERGIC. A mutational analysis of the retention motif in the intact ERGIC-53 is in progress.

H 203 CHARACTERIZATION OF BODIPI AND BODIPY-DERIVATIVE-CONJUGATED-BREFELDIN A. Yuping Deng, Hee Chol Kang*, Jack Bennink, and Jonathan Yewdell. Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD 20892. *Molecular Probes, INC. 4849 Pitchford Ave, Eugene, OR 97402

Three fluorescent analogs of Brefeldin A (BFA) were synthesized. Two of them are isomers of Bodipy conjugated to BFA, and one is a long wavelength bodipy derivative (HCK-IX-52W-F2) conjugated to BFA. These BFA analogs were purified with HPLC and were free of BFA. All of these BFA analogs localized specifically in the endoplasmic reticulum (ER) in many different cell lines. The ER pattern was seen immediately after addition of fluorescent BFA analogs at a concentration as low as 2 ng/ml. The BFA analogs were found to bind to the membrane of the ER. The binding occurred in live cells, fixed cells and BFA resistant cells. Free bodipy did not bind to cells. Compared to BFA, these BFA analogs had similar functions of the BFA but acted slower and less efficient then the BFA. Free bodipy was observed after the bodipy-conjugated BFA were incubated with the cells for two hours. It is likely that these fluorescent anologs were hydrolyzed into free fluorescent probes and BFA, which explains that the fluoresent BFA analogs acted slower and less efficient than the BFA.

H 204 RETROGRADE TRANSPORT OF TOXIN IN THE GOLGI APPARATUS. Kirsten Sandvig, Øystein Garred, Steen H. Hansen* and Bo van Deurs*. Institute for Cancer Research at the Norwegian Radium Hospital, Montebello, 0310 Oslo 3, Norway; *The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark.

It has been suggested that certain protein toxins like the bacterial toxin Shiga toxin as well as some plant toxins like ricin and modeccin are translocated into the cytosol from the endoplasmic reticulum after retrograde transport through the Golgi apparatus. We have recently demonstrated retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum in butyric acid treated A431 cells and shown that the cells under the same conditions become sensitive to the toxin. This sensitization is dependent on protein synthesis during the incubation with butyric acid. Butyric acid treated cells are not intoxicated when Shiga toxin is added at 18 °C, and brefeldin A protects butyric acid-treated cells against the toxin, results that are in agreement with the idea that retrograde transport of Shiga toxin through the Golgi apparatus may be required for intoxication. We have now studied to which extent butyric acid-treated A431 cells are sensitized also to other protein toxins. There is an increased effect of ricin, and the cells become about 100 times more sensitive to modeccin. Also other conditions which are of more physiological importance than butyric acid treatment are able to sensitize A431 cells to Shiga toxin, and we are currently investigating by electron microscopy whether this sensitization is accompanied by retrograde transport.

H 205 THE TRANSMEMBRANE DOMAIN SERVES AS A RETENTION SIGNAL FOR THE GOLGI LOCALISATION OF TWO GLYCOSYLTRANSFERASES.

Paul A. Gleeson, Jo Burke and Rohan Teasdale, Department of Pathology & Immunology, Monash Medical School, Commercial Road, Prahran, Vic., Australia, 3181

The signals reponsible for the localisation of two resident Golgi glycosyltransferases, namely β1,4 galactosyltransferase (Gal T) and N-acetylglucosaminyltransferase I (GlcNAc TI) have been investigated. These membrane enzymes reside in the *trans* and *medial* Golgi compartments, respectively. Like other Golgi glycosyltransferases, Gal T and GlcNAc TI are membrane bound proteins with an N_{in}/C_{out} orientation, containing a short cytoplasmic domain, a dual signal/anchor domain, a short lumenal stem domain and a large lumenal catalytic domain. Species-specific antibodies have been generated to bovine Gal T and rabbit GlcNAc TI, and the expression and localisation of the enzymes have been examined in COS-1 cells and murine L cells transfected with cDNA constructs. The expressed bovine Gal T and rabbit GlcNAc TI were found to be efficiently localised to the Golgi complex in both transfected cells. Using these transfection systems we have explored the molecular basis of the sorting and retention of Gal T and GlcNAc TI to the Golgi complex. From the intracellular localisation of deletion mutants and of hybrid constructs we conclude that a signal contained within the transmembrane domain and flanking residues of the glycosyltransferases specifies compartment-specific Golgi localisation[1,2]. The identification of a hydrophobic, positive retention signal for Golgi proteins suggests a novel mechanism for the localisation of intracellular proteins.

1. Teasdale RD., D'Agostaro G., Gleeson PA. (1992) *J. Biol. Chem.* 267: 4084-96.
2. Burke J, Pettitt, JM, et al., (1992) *J. Biol. Chem.* (in press).

H 206 GPI-ANCHORED PROTEINS AND SPHINGOGLYCOLIPIDS ARE PREFERENTIALLY TARGETED TO THE BASOLATERAL MEMBRANE IN A POLARIZED THYROID EPITHELIAL CELL LINE.

C. Zurzolo#, W. Van 't Hof^, M. Lisanti#, I. Caras*, L. Nitsch", G. van Meer^ and E. Rodriguez Boulan. #Dept of Cell Biol., Cornell Univ. Med. Coll. NY, * Genentech, SF, ^Dept Cell Biol, Utrecht Univ. and "Dpt. Biol e Pat. Cell. e Mol., Napoli Univ., Italy.

The distribution of several apical (DPPIV) and basolateral markers (ßNaK-ATPase, uvomorulin, transferrin receptor, 35-40 Kd Ag, ZO1) in Fisher rat thyroid (FRT) cells is identical to that displayed by these antigens in the model epithelial cell lines MDCK and Caco2 (Zurzolo et al,EMBO, 11:2337, 1992). By a biotin targeting assay, we demonstrated that both apical and basolateral transmembrane proteins were directly targeted to the plasma membrane of FRT cells, indicating that the targeting phenotype of FRT cells was similar to that of MDCK cells. It has been shown that in different kidney and intestinal epithelial cells, both endogenous and exogenous glycosyl-phosphatidylinositol (GPI)-anchored proteins are apically polarized, suggesting a possible role of GPI as an apical targeting signal (Lisanti and Rodriguez-Boulan,TIBS,15:113, 1990). The hypothesis has been proposed that GPI-proteins, and possibly other apical proteins may be introduced into the apical route via clustering with apically targeted glycosphingolipids (van Meer and Simons, J. Cell. Biochem, 36:51, 1988). Unexpectedly we found that the large majority of endogenous GPI anchored proteins of FRT cells are preferentially localized on the basolateral domain, while some of them are apical and some are not polarized. A chimeric GPI-anchored protein (gD1-DAF) formed by the ectodomain of the Herpes glycoprotein gD1 and the DAF signal for GPI-addition was basolaterally targeted in transfected FRT cells, whereas the same fusion protein had been shown to be apically distributed in MDCK cells. Analysis of sphingoglycolipid sorting in FRT cells showed that it was reversed relative to MDCK cells: there was a two- fold greater basolateral targeting of glucosyl-ceramide as compared to SPH. We are led to conclude that clusters of GPI-proteins and glycolipids may be sorted together to the basolateral membrane of FRT cells and suggest that (at least in this cell line) clustering with glycolipids may not have a role in the sorting of trasmembrane apical proteins.Supported by grants from NIH GM 34107 and GM 41771 to ERB.

H 207 STREPTOLYSIN O PERMEABILISED MDCK CELLS PROVIDE A SINGLE *in vitro* SYSTEM TO STUDY THE MULTIPLE PATHWAYS OF POLARISED MEMBRANE TRANSPORT

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Many in vitro systems have been developed that faithfully reconstitute a given step of vesicular transport. Such systems, that have reconstituted both the exocytic and endocytic events, have provided significant insights into the general mechanism of the membrane transport. However, most such systems do not allow to study the process of protein sorting since that necessitates studying more than one process in the same system. To overcome this limitation and since our major interest lies in studying the process of epithelial polarised sorting, we have developed a single system that allows access to multiple steps of transport in one system. We use viral glycoproteins (HA of influenza or G of vesicular stomatits viruses) as the marker proteins. Filter grown MDCK cells are infected with VSV or influenza virus and the viral glycoprotein is allowed to accumulate either in the endoplasmic reticulum or in the *trans*-Golgi network. The apical surface in VSV infected and basolateral surface in influenza infected cells is then selectively permeabilised with the bacterial toxin streptolysin O and the cytosol is washed off. Under these conditions 3 different steps of the biosynthetic pathway can be measured as follows:

1) the ER to Golgi transport by acqusition of Endo H resistense by HA;
2) the TGN to apical transport by trypsin sensitivity of the apically delivered HA ; and
3) the TGN to basolateral transport by surface immunoprecipitation of the transported VSV G.

We show that in our assay conditions all the three transport steps are dependent on temperature, addition of the exogenous cytosol and supply of energy. Currently we are using this system to identify factors that are involved specifically in the apical and basolateral transport.

H 208 LOCALIZATION OF VIP-21, AN INTEGRAL MEMBRANE PROTEIN OF TGN-DERIVED EXOCYTIC VESICLES, TO CAVEOLAE,

Robert G. Parton, Paul Dupree, Teymuras V. Kurzchalia, and Kai Simons, European Molecular Biology Laboratory, Heidelberg, Germany

VIP-21 is a 21Kd integral membrane protein of TGN-derived exocytic vesicles (Kurzchalia et al., J. Cell Biol, 118, 1992). In MDCK epithelial cells, VIP-21 is present in an insoluble complex containing the apically-directed HA protein of influenza virus. In the present study immunofluorescence and immunoelectron microscopy were used to localize VIP-21 in mammalian cells using antibodies raised against specific peptides corresponding to sequences at the C-and N-terminus respectively. Whereas the C-terminal antibody gave a TGN-like staining which co-localized with the HA of influenza virus at 20°C, the antibody against the N-terminus stained only the cell surface by immunofluorescence. By immunoelectron microscopy the N-terminal antibody was shown to label small uncoated invaginations at the plasma membrane, morphologically identical to the structures termed caveolae. To investigate whether these structures are identical to the plasmalemmal vesicles described in endothelial cells, thin frozen sections were labelled with antibodies against the N-terminus. Plasmalemmal vesicles were heavily labelled. Uncoated invaginations have also been implicated in the internalization of glycolipid-binding toxins. The binding sub-unit of cholera toxin (CT-B), which specifically binds to the ganglioside GM1, was adsorbed to colloidal gold. CT-B-gold was concentrated in non-coated invaginations of A431 cells at 4°C and colocalized with VIP-21 (N). In addition, in the GM1-deficient cell line, BHK, cholera toxin binding could be reconstituted by addition of exogenous GM1. In this reconstituted system, CT-B-gold was again shown to be concentrated in VIP-21(N)-positive caveolae. Caveolae may be involved in the recycling of VIP-21 to the TGN. In addition, caveolae may be sites of clusters or rafts of glycosphingolipids analogous to those postulated to be involved in sorting apical proteins in the TGN of epithelial cells.

H 209 *DIFFERENTIAL INHIBITION OF SEQUENTIAL TRANSPORT STEPS BETWEEN THE ER AND TGN IN VITRO*, Howard W. Davidson and William E. Balch, Departments of Cell and Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037.
We have developed a system capable of measuring vesicular transport from the endoplasmic reticulum to the trans Golgi network in perforated NRK cells. Movement from the ER to the cis Golgi compartment is assessed by the conversion of the tsO45 VSV glycoprotein (VSV-G) from a totally endoglycosidase D (endo D) resistant form, to a species containing one endo D resistant and one endo D sensitive oligosaccharide. Delivery to the medial cisternae is measured by the appearance of the completely endo D sensitive form of VSV-G, or by the acquisition of complete resistance to endo H, and delivery to the TGN by the appearance of an endo H resistant form of VSV-G which is sensitive to digestion with neuraminidase.

Movement between each sequential compartment requires ATP and cytosol, and is inhibited by GTPγS and inhibitory antibodies towards NSF. In contrast, a mutant form of the small molecular weight GTP-binding protein rab1A (Asn^{124}→Ile) inhibits movement between the ER and cis Golgi, and between the cis and medial cisternae, but does not affect transport from the medial Golgi to the TGN. Conversely, the protein kinase inhibitor staurosporine prevents movement between the medial Golgi and the TGN, but does not influence transport between the ER and early Golgi compartments. This system should enable us to more accurately define the roles of individual transport components, including the various members of the rab gene family, in trafficking between compartments of the exocytic pathway.

H 210 *SORTING SIGNALS WITHIN THE CYTOPLASMIC TAIL OF THE MHC ASSOCIATED INVARIANT CHAIN.*

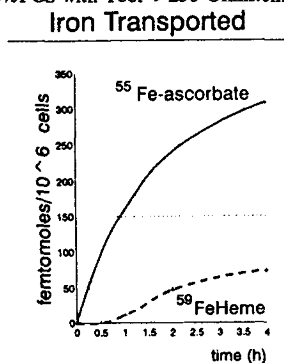
Oddmund Bakke, Bjørn Bremnes, Hege Hardersen and Toril Madsen. Division of Molecular Cell Biology, Department of Biology, University of Oslo, Norway.

The human MHC class II associated invariant chain (Ii) is a type II membrane protein which is sorted to endosomal compartments. Transient and stable transfections of Ii with deletions from the cytoplasmic tail in several cell lines have shown that endosomal sorting information is contained within this region of the molecule. Neuraminidase (NA) from influenza virus, is a type II plasma membrane protein which is not found in endosomal compartments. Fusion proteins containing NA and parts of the cytoplasmic tail of Ii were constructed and expressed transiently in CV1 cells. By using antibodies towards NA we found that molecules containing either the first 11 amino acids of the Ii tail or the remaining 19 amino acids were also expressed on the plasma membrane and efficiently internalized to early and late endosomes and lysosomes. The first signal contain an LI motif and when this was mutated, the fusion protein was not seen in endosomes. Likewise, removal of the first 4 amino acids of the second signal (EQLP) greatly reduced internalization, and the protein was not detected in endosomes. This show that the cytoplasmic tail of the invariant chain contain two independent internalization/sorting signals which may route molecules to the endocytic pathway.

H 211 ^{59}Fe-Heme and ^{55}Fe-ascorbate transport kinetic reflect different compartmentalization in Caco-2 cells.

Xavier Alvarez, Roya Mansouri and Jonathan Glass. LSUMC, Heme/Onco, Shreveport, LA, 71130

Caco-2 cell monolayers on porous membranes in bicameral chambers grown in DMEM-10%FCS with Teer >250 Ohms.cm^2 were used to study the transport of Fe from heme (apical chamber; 1uM ^{59}Feheme:BSA, 1:3 molar ratio) and inorganic Fe (^{55}Feascorbate, 1:1000). Basolateral (Bl) medium was made 50uM in apoTf. Fig 1 shows the appearance of Bl Fe. The Fe from heme detected in the Bl partitioned into water rather than into cyclohexanone, suggesting heme was cleaveged and the inorganic Fe released in order to be transported. Apparently the catabolism of the heme ring is an obligated step in the transport of it's Fe. Activity of Heme Oxygenase (HO), 5.5±2 pmoles of bilirubin/min/mg of cell protein was detected in the Caco-2 cells. As HO activity is located mainly to the cell microsomal fraction, the transport kinetics suggest that Fe in heme moiety is compartmentalized (to microsomes) to be degraded and then the Fe joins the inorganic Fe transport pathway in the Caco-2 cells.



H 212 REGULATION OF TRANSCYTOSIS OF THE POLYMERIC IMMUNGLOBULIN RECEPTOR IN MDCK CELLS BY PROTEIN KINASE-C. M. H. Cardone and K. E. Mostov

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We observed that phorbol myristate acetate (PMA) stimulates transcytosis of the polymeric immunoglobulin receptor (pIgR) in Madin-Darby canine kidney (MDCK) cells. Apical release of pre-endocytosed ligand to the pIgR (Fab fragments of polyclonal antibodies against the ectodomain of pIgR) can be stimulated by 15% over a 2 hour time course, with an initial increase seen in less than 10 minutes. In addition, apical surface delivery of pIgR and cleavage of its ectomain to secretory component (SC) is also stimulated by PMA. This effect of PMA suggests that protein kinase C (PKC) is involved in the regulation of pIgR trafficking in MDCK cells. To test this, we down regulated PKC activity by pre-treating cells with PMA for 16 hours and observed that transcytosis could no longer be stimulated. The PKC specific inhibitors H-7 and calphostin prevent PMA from stimulating transcytosis of the Fab ligand, further demonstrating the involvement of PKC. In addition, calphostin causes a decrease in the basal level of transcytosis of pre-internalized ligand. In an attempt to localize the region of the pIgR which is acted upon by PKC we have tested several mutant receptors for stimulation by PMA. Mutants of pIgR in which serines at positions 664, 725, or 726 are replaced by alanine are stil able to be stimulated to transcytose by PMA.

H 213 IDENTIFICATION OF A NOVEL GOLGI PROTEIN CONTAINING A CYTOPLASMIC DOMAIN OF GREATER THAN 300KD. Adam Linstedt and Hans-Peter Hauri, Deptartment of Pharmacology, Biocenter of the University of Basel, CH-4056 Basel , Switzerland.

We have generated a mAb, G1/133, using a Golgi fraction isolated from the human intestinal cell line CaCo-2 as the immunogen. The mAb recognizes an epitope specific to the Golgi apparatus since in double labeling experiments the G1/133 yielded an immunofluorescence pattern nearly indistinguishable from that obtained with an antibody against a known Golgi protein, galactosyltransferase. Furthermore, both the G1/133 and galactosyltransferase patterns were similarly affected at various times after addition of okadaic acid or brefeldin A, agents that disrupt Golgi structure. The epitope appears to be conserved across mammalian species and present in most cell types as G1/133 yielded a Golgi pattern in all cell lines tested. A protein with an apparent molecular weight of greater than 400kD was immunoprecipitated and immunoblotted with G1/133. The antigen was recovered from membrane fractions after differential centrifugation or sucrose flotation and was not released from the membrane by carbonate treatment. Thus, the protein appears to be an integral component of the Golgi membrane and may have a disulfide linked lumenal domain because it exhibited reduced mobility on non-reducing SDS gels. Strikingly, the majority of the protein including the G1/133 epitope is cytoplasmically disposed since G1/133 did not react with protease digested membranes, but did recognize a soluble proteolytic fragment with an apparent molecular weight of greater than 300kD. This very large cytoplasmic domain may indicate that the protein functions in maintaining or regulating the structure of the Golgi.

H 214 ASSOCIATION OF SMALL GTP-BINDING PROTEINS WITH SECRETORY GRANULES IN CYTOTOXIC T LYMPHOCYTES. Witte Koopmann, Lukas Huber, Kai Simons, and Yair Argon, Department of Immunology, Duke University Medical Center, Durham, NC 27710 and EMBL, Heidelberg, Germany

The exocytosis of specialized granules by cytotoxic T lymphocytes (CTL) is a major mechanism by which the cells kill their targets. Despite the importance of this process for proper function of the immune system, very little is known regarding the biochemical mechanisms which regulate it. Small GTP-binding proteins control various steps in protein trafficking along both the endocytic and exocytic pathways, including ER to Golgi transport and intra-Golgi transport. However, direct evidence for the involvement of such proteins in exocytosis, the final stage in vesicular protein transport, has been lacking. We therefore sought to examine the possible involvement of small GTP-binding proteins in CTL exocytosis. CTL were prepared from mouse spleen by culturing CD8+ splenocytes for 5-7 days in the presence of interleukin-2 and conditioned medium. CTL granules were purified on Percoll gradients and subjected to two-dimensional gel electrophoresis, transfer to nitrocellulose, and blotting with $\alpha^{32}P$-GTP. At least seven small GTP-binding proteins were identified in the granule fraction by this method, including rab5, rab7, rab8, rab10, and rab11.

To further examine which small GTP-binding proteins might be involved in CTL exocytosis, a polymerase chain reaction-based strategy was employed. A degenerate oligonucleotide primer pair was designed to the conserved WDTAGQ and GNKXD motifs common to all small GTP-binding proteins; these primers were used in a PCR reaction using CTL cDNA as template. Subcloning and sequencing of the 189bp products obtained from such a reaction revealed that a large number of small GTP-binding protein mRNAs are expressed in CTL. The sequences confirmed to date include rab8, rab10, and rab11, identified by homology with previously described cDNA sequences. The levels of identity of the mouse clones to their corresponding sequences in the GENBANK/EMBL databases range between 78 and 95 %. Analysis of rab11 expression by both PCR and Northern hybridization showed that rab11 is strongly induced during the differentiation of resting splenocytes into CTL. We are currently examining the induction of additional rab mRNAs, and pursuing the functional role of rab proteins in CTL exocytosis.

H 215 RECONSTITUTION OF ENDOPLASMIC RETICULUM ASSEMBLY *IN VITRO*, Jeffry D. Watkins and William E. Balch, Dept. of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037

We introduce a new assay to quantitatively measure the fusion of endoplasmic reticulum (ER) vesicles *in vitro*. This assay takes advantage of the unique environment of the ER that allows for the proper folding, disulfide bond formation, and oligomerization of newly formed proteins. Partially purified ER membranes were obtained from two mutant myeloma cell lines derived from the same parent line. One mutant line, P3X63Ag8U.1 (P3U.1), expresses the mouse immunoglobulin K light chain in the absence of heavy chain, while the other line, Ag8.(8), synthesizes the mouse immunoglobulin γ_1 heavy chain in the absence of light chain. Both cell lines retain their respective immunoglobulin subunits in the ER. We have established incubation conditions which support fusion of ER vesicles isolated from Ag8.(8) (heavy chain only) with vesicles isolated from ^{35}S-labeled P3U.1 (light chain only) cells. Following membrane fusion and mixing of the lumenal contents, efficient oligomerization of the heavy and light chains to form mature immunoglobulin G was observed. Membrane fusion/oligomerization was time- and temperature-dependent and required ATP. Efficient fusion/oligomerization required membrane-associated proteins which were sensitive to trypsin and the non-hydrolyzable analog of GTP, GTPγS. The development of a rapid and quantitative assay to measure the assembly of the ER *in vitro* will allow us to identify factors involved in establishing and maintaining the structure of the ER *in vivo*.

H 216 CLONING AND CYTOPLASMIC EXPRESSION OF ANTIBODIES DIRECTED AGAINST *RAB2*
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Rab2 is one of two *ras*-like low molecular weight GTP binding proteins that have been localised to a compartment intermediate between ER and Golgi (an intermediate compartment, "IC"). The other, *Rab1b*, has been shown to play a role in anterograde transport through the exocytic pathway. Neither antibodies to *Rab2* nor synthetic peptides derived from *Rab2* appear to inhibit these transport assays. However, there is increasing evidence that the IC is not a simple vectorial intermediate but rather a branch point akin to the TGN, so there are a variety of possible transport steps that could involve *Rab2*.

In order to study the function of *Rab2* in the IC we have prepared a panel of monoclonal antibodies against the purified recombinant protein and against synthetic peptides corresponding to the hypervariable C terminal region that is important for intracellular localisation. Several of these mabs have now been cloned and sequenced. The V_H and V_L regions from the mabs have been assembled into single domain Fv constructs modified for cytosolic expression and carrying an epitope tag. Expression of this recombinant molecule in mammalian cell lines under a steroid inducible promoter is now being used to examine effects on the morphology of the cells, the morphology of the IC, ER and Golgi, and the transport of viral glycoprotein reporter molecules.

In parallel, a series of point mutations have been introduced into the *Rab2* coding sequence, and the resulting constructs are being expressed in bacteria so that the GTP binding and hydrolysis activity of the mutant proteins may be measured. Expression of these recombinant molecules in mammalian cell lines under a steroid inducible promoter is also being used to search for trans dominant effects on the morphology of the cells, the morphology of the IC, ER and Golgi, and on viral glycoprotein transport.

H 217 INVOLVEMENT OF A CYTOSOLIC PROTEIN COMPLEX IN PROTEIN EXPORT FROM THE ENDOPLASMIC RETICULUM, Frank J. Peter, Helen Plutner, and William E. Balch, Dept. of Cell Biology, Scripps Clinic Resch. Fndn., La Jolla, CA 92037

We have recently developed a morphological assay to investigate intermediate steps in vesicular traffic from the endoplasmic reticulum (ER) to the Golgi complex in vitro (Plutner et al., *J. Cell Biol.*, in press). Using indirect immunofluorescence we demonstrated that newly synthesized VSV-G initially enters a punctate intermediate defined by the p58 antigen. This step requires both cytosol and ATP.

We have now begun to identify those components which are required to support protein transport from the ER to the p58 intermediate. We have separated rat liver cytosol (rlc) by gel filtration chromatography and identified a high molecular weight complex (>1000kD) which supports the export of protein from the ER. In contrast, complete transport to the cis Golgi requires additional components found to fractionate at lower molecular weights. Preliminary analysis of the complex indicates that it contains a number of distinct proteins, including rab1, a protein previously implicated to be essential for ER to Golgi transport. We are currently investigating the identity of other components in the complex and the possible interactions between them.

H 218 *INHIBITION OF INTRA-GOLGI TRANSPORT IN VITRO* BY MITOTIC KINASE, Deborah J.G. Mackay, Rosemary A. Stuart, Jörg Ademczewski and Graham B Warren, Cell Biology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, UK

The exocytic and endocytic pathways are inhibited at multiple sites during mitosis in mammalian cells. In order to investigate transport through the Golgi complex during mitosis, an *in vitro* assay was used to reconstitute the intra-Golgi transport of the vesicular stomatitis virus G-protein. Transport of VSV-G between Golgi cisternae was measured using purified Golgi membranes supplemented with interphase or mitotic cytosol extracts from HeLa cells, and mitotic activity was measured by histone kinase assay. The assay shows a 75% inhibition of intra-Golgi transport by mitotic cytosol made from metaphase-arrested HeLa cells. A similar inhibition was caused by interphase cytosol activated by addition of recombinant cyclin A. Inhibition of intra-Golgi transport and activation of histone kinase activity were completely blocked by staurosporine (a general kinase inhibitor), or by reduction in ATP levels. The transport inhibitory activity is a cytosolic factor, and its activation appears to be reversible in the transport assay. This *in vitro* system therefore provides an opportunity to investigate the mechanism of mitotic inhibition of intra-Golgi transport, and possibly of the general inhibition of membrane traffic in mitotic cells.

H 219 Coatomer, ARF, and a Positively-Acting Factor in Golgi Transport.

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Purified coatomer and recombinant myristoylated ARF will form coated vesicles from Golgi membranes in the absence of other cytosolic proteins (Orci, Palmer, and Rothman, unpublished). The combination of coatomer and myristoylated ARF inhibits transport reconstituted with a mixture of purified (p115, SNAPs, NSF) and partially purified fractions (Iα and Iβ) cytosolic components, but this combination does not inhibit transport reconstituted with crude cytosol.This difference suggests that a factor present in crude cytosol (but missing among the purified purified cytosolic fractions) is required to complete transport from coated buds and/or coated vesicles. We propose that in the absence of this factor, only a partial reaction would occur in which coated structures are produced but can not be consumed. We describe the resolution and partially purification of such a factor.

ARF may play a dual role in Golgi transport. While only the myristoylated ARF can (with coatomer) inhibits transport, both non-myristoylated and myristoylated ARF, can each stimulate transport when other cytosolic components are limiting. Transport stimulated by ARF required both ATP and GTP, and was inhibited by Brefeldin A.

H 220 THE ROLE OF SMALL GTPases IN β_2-ADRENERGIC RECEPTOR SEQUESTRATION, B.F. Dickey, Y. Srinivasan, F.O. Levy, L. Birnbaumer and B.J. Knoll, Departments of Pulmonary Medicine and Cell Biology, Baylor College of Medicine, Houston, TX 77030

Multiple small GTPases are involved in regulating vesicle traffic in eukaryotic cells. Two of these, Rab4 and Rab5, are associated morphologically with early endosomes in vivo, and Rab5 is required for the fusion of early endosomes in vitro. Rab7 has been shown to colocalize with late endosomes. Endosomes are likely to be the compartments into which β_2-adrenergic receptors (β_2ARs) move shortly after binding to agonist. The movement of β_2ARs into vesicles (sequestration) makes them inaccessible to binding by hydrophilic ligands, while they are still able to bind hydrophobic ligands. Rabs 4, 5 and 7 are being studied to further our long range goal of understanding the route and mechanisms of endocytosis of the large family of seven-membrane spanning G-protein coupled receptors. We have constructed a line of mouse Ltk- cells (Ltk-hβ_2AR) that expresses human β_2AR at a level of 300,000 receptors per cell. Treatment of these cells with the β-adrenergic agonist isoproterenol for 5' results in 40-50% of receptors becoming inaccessible to the hydrophilic ligand CGP12177, while the total number of cellular receptors remains unchanged, as assessed by binding to the hydrophobic ligand dihydroalprenolol. Dominant suppressor mutations of Rabs 4 and 5 are being constructed and expressed in the Ltk-hβ_2AR line to examine their effect on agonist-induced sequestration of receptors, and on the eventual return of receptor to the surface. Rab7 also may be involved in the downregulation of β_2ARs which occurs after prolonged (>3 hrs) exposure to agonist, an event that is at least partly due to receptor degradation. Studies are in progress that will examine the association of Rabs 4,5 and 7 with vesicles containing sequestered β_2AR from short and long-term agonist-treated cells.

H 221 GTPγS INHIBITS ORGANELLE TRANSPORT ALONG AXONAL MICROTUBULES; George S. Bloom, Bruce R. Richards, Philip L. Leopold, Donna M. Ritchey and Scott T. Brady; Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center; Dallas, TX 75235

Movements of membrane-bounded organelles through cytoplasm frequently occur along microtubules, as in the neuron-specific case of fast axonal transport. To shed light on how microtubule-based organelle motility is regulated, pharmacological probes for GTP-binding proteins, or protein kinases or phosphatases were perfused into axoplasm extruded from squid (*Loligo pealei*) giant axons, and effects on fast axonal transport were monitored by quantitative video-enhanced light microscopy. GTPγS caused concentration-dependent and time-dependent declines in organelle transport velocities. GDPβS was a less potent inhibitor. Excess GTP, but not GDP, masked the effects of co-perfused GTPγS. The effects of GTPγS on transport were not mimicked by broad spectrum inhibitors of protein kinases (K-252a) or phosphatases (microcystin LR, and okadaic acid), or as shown earlier, by ATPγS. Therefore, suppression of organelle motility by GTPγS was guanine nucleotide-specific, and evidently did not involve irreversible transfer of thiophosphate groups to protein. Instead, the data imply that organelle transport in the axon is modulated by cycles of GTP hydrolysis and nucleotide exchange by one or more GTP-binding proteins. Fast axonal transport was not perturbed by AlF_4^-, indicating that the GTPγS-sensitive factors do not include heterotrimeric G-proteins. Potential axoplasmic targets of GTPγS include dynamin and multiple small GTP-binding proteins, which were shown to be present in squid axoplasm. These collective findings suggest a novel strategy for regulating microtubule-based organelle transport and a new role for GTP-binding proteins. Supported by NIH grants NS23868 and NS30485, and Robert A. Welch Foundation grant I-1077.

H 222 REGULATION OF MEMBRANE TRAFFICKING BY FATTY ACYLATION D.I. Mundy, Dept. of Neurology, Baylor College of Medicine, Houston, TX 77030

It has been suggested that reversible fatty acylation is required for the budding and fusion of transport vesicles in a cell-free system that reconstitutes intra-cisternal transport in the Golgi apparatus, however, the fatty acylated protein(s) has not been identified. Since vesicular transport is inhibited during mitosis, I reasoned that a comparison of the fatty-acylated proteins in mitotic cells with those in interphase cells might be useful in identifying the acylated protein(s) involved in the fusion process. By comparing the acylated proteins from mitotic and interphase cells I discovered that a 62kD protein is selectively palmitoylated during mitosis. (JCB 116:135-146; 1992). The fungal metabolite, brefeldin A (BFA) also inhibits inter-compartmental transport of proteins, and causes the dramatic disappearance of the Golgi apparatus in most cell types. Strikingly, the same protein that is acylated during mitosis becomes acylated within minutes, in BHK, CHO, HeLa, NRK and A431 cells treated with BFA. PtK1 cells are resistant to the effects of BFA on the Golgi apparatus but remain sensitive to the effects of BFA on endocytic organelles. In this cell line p62 is not acylated by BFA, indicating that the effect on the acylation of p62 is specific for the early part of the pathway. This is consistent with data on the subcellular fractionation of p62 which indicates that this protein resides in the intermediate compartment or in the cis Golgi. p62 may be involved in ER to Golgi transport and to examine this I have reconstituted the the acylation of p62 in permeabilized cells. Acylation is dependent on the addition of both cytosol and ATP. In addition, the acylation of p62 is blocked by GTP-γ-S and Al/F which is consistent with the involvement of p62 in vesicular transport. I am currently producing antibodies to p62 to further address its function in membrane trafficking.

H 223 IDENTIFICATION OF *N*-ETHYLMALEIMIDE-SENSITIVE FUSION PROTEIN AS A COMPONENT OF SYNAPTIC VESICLES, Mitsuo Tagaya, Yoshinori Moriyama, Akitsugu Yamamoto, and Yutaka Tashiro, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567 and Department of Physiology, Kansai Medical University, Moriguchi, Osaka 570, Japan

N-ethylmaleimide-sensitive fusion protein (NSF) was purified as the factor that mediates vesicular transport between the Golgi cisternae (Block, M. R., Glick, B. S., Wilcox, C. A., Wieland, F. T., and Rothman, J. E. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 7852-7856). Immunoblot analysis by using monoclonal antibodies against *Escherchia coli*-produced Chinese hamster ovary NSF has revealed that a protein similar to NSF (p76) is copurified with synaptic vesicles, the organella that undertake the secretion of neurotransmitters. p76 was not released from synaptic vesicles by incubation with Mg^{2+}-ATP, the condition where NSF associated with Golgi membranes is released. Anionic detergents such as deoxycholate were required for the solubilization of p76 from synaptic vesicles. Immunoblot analysis of other secretory organella has shown that a protein similar to NSF associates not only with synaptic vesicles, but also with microvesicles and large dense-core vesicles of bovine pituitary, and chromaffin granules of bovine chromaffin cells. These results suggest that NSF is employed as a general apparatus for exocytosis.

H 224 *IN VITRO* RECONSTITUTION OF THE pH-DEPENDENT AGGREGATION OF PANCREATIC ZYMOGENS. INVOLVEMENT OF GP-2 IN THE PROCESS, D. LeBel, G. Viau, J. Lainé, and F.A. Leblond. Centre de Recherche sur les Mécanismes de Sécrétion, Université de Sherbrooke, Sherbrooke, Canada, J1K 2R1.

Regulated secretory proteins are thought to be sorted in the *trans*-Golgi network (TGN) via selective aggregation. To elucidate the biogenesis of the secretory granule in the exocrine pancreas, we reconstituted *in vitro* the conditions of pH and ions believed to exist in the TGN using the end product of this sorting process, the content of mature zymogen granules. In this reconstituted system, protein aggregation was dependent on pH (acidic) and on cations (10 mM Ca^{2+}, 150 mM KCl) to reproduce the pattern of proteins found in the granule. The constitutive secretory protein IgG was excluded from these aggregates, showing that the system was specific for regulated secretory proteins. An additional factor was found to be important in the system, that is the relative proportion of the major granule membrane protein GP-2 in the assay. Zymogens aggregation indeed correlated perfectly with its specific activity expressed in µg of GP-2 per mg of zymogens. GP-2 did also function with parotid granule contents. These data show that the glycosyl phosphatidylinositol anchored protein GP-2 coaggregates with zymogens in an acidic environment similar to the one believed to exist in the pancreatic TGN, and thus suggest that GP-2 could function as a membrane anchor for zymogen aggregates, facilitating their entrapment in budding vesicles directed towards the regulated secretory pathway.

Supported by grants from the Canadian Cystic Fibrosis Foundation and NSERC of Canada.

H 225 BINDING OF COAT PROTEINS TO GOLGI AND TO ENDOSOMES IN INTACT CELLS AND IN VITRO, J. Andrew Whitney and Ira Mellman, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510

Recent work by several labs has implicated cytosolic 'coat' proteins in the regulation of intracellular membrane transport. We have extended our previous work on the binding of Golgi coatomer proteins in MDCK cells and have suggestive evidence for the existence of similar coat proteins interacting with endosomal membranes.

Previously, we have shown that treatment of MDCK cells with brefeldin A (BFA) at 37°C blocks transcytosis of IgA and causes the tubulation of endosomes while leaving Golgi morphology and binding of β-COP unaffected. Interestingly, we find that β-COP binding to Golgi in MDCK cells becomes sensitive to BFA when the cells are incubated at 20°C. This effect is seen within 5-10 min of BFA addition and can be reversed by raising the temperature from 20°C to 37°C, even in the presence of BFA.

Given the endosomal sensitivity to BFA at both temperatures, we established an in vitro assay to identify elements of a putative BFA-sensitive endosomal coat complex. Our approach involves incubation of ^{35}S-labeled cytosol with target membranes followed by centrifugation, SDS-PAGE and transfer to nitrocellulose. In this way, we can analyze a gel for both known proteins (by immunoblotting) and unknown proteins (by ^{35}S autoradiography). To assess the feasibility of this method, we used CHO Golgi-enriched membranes to demonstrate BFA sensitivity, GTPγS and AlF_n^- stimulation of β-COP binding. As expected, binding of β-COP to MDCK Golgi at 37°C is insensitive to BFA but is stimulated by GTPγS. By ^{35}S autoradiography, we demonstrate binding of a set of proteins corresponding in size to the major elements of the Golgi coat complex, and have found that AlF_n^- stimulates the binding of two additional proteins of ~75 kD and ~34 kD. Similar proteins bind to CHO Golgi, except that binding is BFA-sensitive particularly in the presence of impermeant reducing agents.

Preliminary experiments using highly purified CHO cell endosomes prepared by free-flow electrophoresis show a restricted set of proteins whose binding is enhanced by GTPγS and inhibited by BFA. Current work is directed towards determining whether or not these proteins represent an endosomal coat complex.

H 226 INTRACELLULAR LOCALIZATION OF MHC CLASS II MOLECULES AND ASSOCIATED INVARIANT CHAIN.

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In antigen presenting cells the MHC class II molecules associate with invariant chain (Ii) in ER. This complex is transported to intracellular compartments where the class II molecules bind antigen while Ii is degraded. Using light and electron microscopy (EM) combined with immunocytochemical labelling we have characterized the intracellular localization of Ii and MHC class II molecules in stably transfected human fibroblasts. The labelling was combined with the localization of endocytic markers. Immunofluoresence studies show that both Ii and class II localize mainly in small vesicles with a clear surface labelling for class II. In some of the cells the proteins localize in large, vacuolar like compartments. Similar compartments are seen in cells transfected with Ii alone, but not with class II alone. Immunocytochemical labelling for EM show that the small Ii - class II positive vesicles represent early as well as late endosomes while the large Ii - class II positive compartments consist of a complex nettwork of membranes surrounding a large lumen with no visible content. Double labelling for class II and Ii reveal that some large compartments label for both class II and Ii, others label for class II only. Most of the large compartments contain the mannose-6-phosphate receptor. Endocytosed BSA-FITC is seen in large compartments after 10 min. After longer chase periods the endocytosed marker occour in LAMP positive vacuoles. Together these studies show that MHC class II proteins and the associated Ii intracellularly localize in both early and late endosomes and in the special large compartments.

H 227 COMPLEMENTATION ANALYSIS OF CHEDIAK-HIGASHI SYNDROME: EVIDENCE THAT THE SAME GENE IS RESPONSIBLE FOR THE DEFECT IN ALL PATIENTS AND SPECIES EXAMINED, Charles M. Perou and Jerry Kaplan, Division of Cell Biology and Immunology, Department of Pathology, University of Utah, Salt Lake City, UT 84132.

Chediak-Higashi Syndrome (CHS) is an autosomal recessive disease of humans which results in defective vesicle formation. A distinctive phenotypic feature of this disorder is the presence of "giant" lysosomes, which tend to cluster near the nucleus. These enlarged lysosomes are thought to result from repeated, inappropriate lysosome to lysosome fusions. The Beige mouse and the Aleutian mink are believed to be animal homologues of CHS based solely upon phenotypic similarities. We have utilized a cell fusion assay which suggests that the same gene is defective in all patients and species examined. One set of cells were incubated in Lucifer Yellow containing medium, and another set incubated in Dextran-Texas Red, in order to visualize lysosomes. When wild type cultured fibroblasts from any species (ie. human, mouse, or mink) were fused (via UV inactivated-Sendai virus) to mutant cells from any species, the resulting heterokaryon's lysosomes were phenotypically normal with respect to lysosome size and distribution. Fusion of mutant (ie. mouse, mink, or three different human CHS isolates) to mutant did not affect the CHS phenotype of large, perinuclear lysosomes. These results suggest that the lack of complementation seen in the mutant-mutant fusions are due to a mutation within the same gene.

H 228 SORTING OF THE MANNOSE - 6 - PHOSPHATE RECEPTORS, Fabienne Mauxion, Stéphane Méresse, Anne Schmidt and Bernard Hoflack, Department of Cell Biology, European Molecular Biology Laboratory, 6900 Heidelberg, Germany.

In higher eucaryotic cells, the soluble lysosomal enzymes are targeted to lysosomes by a mechanism involving two related mannose-6-phosphate receptors: the cation-independent MPR (CI-MPR) and the cation-dependent MPR (CD-MPR). The carboxy-terminal end of the CI-MPR cytoplasmic tail is required for efficient targeting of lysosomal enzymes. We have previously shown that two highly conserved sequences within this region could be phosphorylated *in vivo* by a kinase associated with the Golgi-specific adaptator HAI which participates in the formation of the Golgi-derived clathrin-coated vesicles. Acquisition of phosphate groups correlated with exit of the CI-MPR from the Trans-Golgi Network while dephosphorylation occurred likely in endosomal compartments.

To investigate whether the cytoplasmic domain of the CD-MPR contains similar sorting signals, we have fused the luminal domain of the influenza virus hemagglutinin to the transmembrane and cytoplasmic domains of the CD-MPR. This fusion protein, when expressed into cell lines, is localized in early and late endosomal compartments demonstrating that the cytoplasmic tail of the CD-MPR contains sufficient information for endosomal targeting. Deletion of the 16 last residues of the CD-MPR cytoplasmic tail induces an increase in the cell surface localization of the chimeric protein. Interestingly, the CD-MPR cytoplasmic tail presents in this deleted region a sequence similar, although not identical, to the phosphorylation sites of the CI-MPR. However, the CD-MPR is not phosphorylated *in vivo* as well as *in vitro*. By a single point mutation, we have introduced a typical phosphorylation site in the cytoplasmic tail of the HA/CD-MPR chimera. The resulting mutant fusion protein showed a different steady-state distribution from that of the wild-type fusion protein, being mostly localized in late endosomes. We speculate thus that the phosphorylation is required for targeting to late endosomes.

H 229 TEMPERATURE-SENSITIVE REDISTRIBUTION AND ENDOCYTOSIS OF ΔF508CFTR IN TRANSFECTED CELLS, Gergely L. Lukacs, Xiu-Bao Chang, Norbert Kartner, Christine E. Bear, John R. Riordan, Sergio Grinstein, Hospital for Sick Children, Toronto, Canada.

Cystic fibrosis (CF) is associated with a defective epithelial Cl^- conductance and is caused by mutations in the cystic fibrosis conductance transmembrane regulator (CFTR), a plasma membrane Cl^- channel regulated by protein kinase A (PKA). The most common mutation in CF is a deletion of phenylalanine 508 (ΔF508CFTR), that may result in defective channel activity and/or mislocalization of the protein. To study the mechanism underlying defective Cl^- permeability in CF we have stably transfected Chinese hamster ovary (CHO) cells with the ΔF508CFTR cDNA. The plasma membrane Cl^- conductance was estimated by fluorescence and electrophysiological techniques. Localization of CFTR was assessed by subcellular fractionation and Western blotting. PKA-activated plasmalemmal Cl^- conductance and the mature (fully glycosylated) form of ΔF508CFTR were not detectable in cells grown at 37°C. In contrast, after culturing the cells at <29°C, PKA activated Cl^- permeability and immunoreactive ΔF508CFTR appeared in the plasma membrane. To study the destination of the mutant CFTR after reaching the plasma membrane, a method was implemented to measure the ionic conductance of intracelluar compartments *in situ* (J. Biol. Chem. [1992] 267: 14568). ΔF508CFTR is internalized to the endosomal compartment, disappearing from the plasma membrane with a half-life of ~4 h. The kinetics of internalization of wild type and mutant CFTR was compared and will be discussed.

H 230 RECEPTOR-MEDIATED ENDOCYTOSIS OF EGF *IN VITRO*: DIFFERING REQUIREMENTS FOR LIGAND-INDUCED VS CONSTITUTIVE INTERNALIZATION, Christophe Lamaze, Thomas E. Redelmeier, Takeshi Baba and Sandra L. Schmid, Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, 92037

Receptors internalized via coated pits fall into two classes: those which are constitutively internalized, such as the transferrin-receptor (Tfn-R), and those whose internalization is ligand-induced, such as the epidermal growth factor receptor (EGF-R). We have recently extended our studies on Tfn-R internalization and developed a cell-free assay to measure EGF-R mediated endocytosis in perforated A431 cells. Using stage-specific assays which distinguish the sequestration of ligands into deeply invaginated coated pits from the internalization of ligands into sealed coated vesicles, we find that EGF sequestration *in vitro* is efficient and occurs at >80% the rate and extent of sequestration in intact cells. However, we cannot detect internalization of EGF in our system, consistent with our previous findings that internalization of Tfn receptors *in vitro* occurs only through budding coated pits formed *in vivo*. While constitutively internalized Tfn-R populate coated pits even in the absence of ligand, unoccupied EGF-R are not localized to coated pits. As for Tfn, EGF sequestration requires ATP hydrolysis and cytosol and is inhibited by GTPγS. We have also observed the following biochemical differences between ligand-induced EGF-R sequestration and constitutive internalization of Tfn-R. 1) ATP- and cytosol-independent partial reactions observed for Tfn sequestration are not observed for EGF sequestration. Together with our inability to detect EGF internalization *in vitro*, these results provide the first evidence to suggest that EGF binding does not induce receptors to migrate into pre-existing coated pits, but instead triggers *de novo* coated pit assembly. 2) Purified adaptor molecules (APs) which stimulate Tfn sequestration *in vitro* do not stimulate EGF sequestration. 3) Bovine brain cytosol which supports Tfn internalization into perforated A431 cells does not support high efficiency EGF sequestration but human K562 cell cytosol does. 4) EGF sequestration is inhibited by tyrosine kinase inhibitors, while Tfn sequestration is resistant to these.

We are currently investigating the molecular basis for these differing biochemical requirements for EGF sequestration. Using HRP and gold-conjugated ligands we are also using morphological techniques to analyze the pathways of EGF and Tfn sequestration *in vitro*. These studies should allow us to further characterize growth factor signalling transduction and to identify new components necessary to this process.

H 231 GENETIC AND *IN VITRO* ANALYSIS OF END4 MUTANTS OF CHINESE HAMSTER OVARY CELLS, Rockford K. Draper, Ru-Hung Wang and Penelope A. Colbaugh, Biology Program, The University of Texas at Dallas, Richardson, TX 75083.

The End4 complementation group of Chinese hamster ovary (CHO) cell mutants was defined originally with mutant V.24.1, a temperature-sensitive mutant selected for resistance to diphtheria toxin. At the restrictive temperature, secretion by V.24.1 cells is blocked between the endoplasmic reticulum and the Golgi and the Golgi complex disappears. The mutant cells also express a 50% reduction in fluid-phase endocytosis. We recently devised a new method for isolating mutants deficient in fluid-phase endocytosis, based on the observation that endocytosed horseradish peroxidase could be made lethal to cells. One CHO cell mutant isolated with this method, termed mutant HRP-1, had properties similar to V.24.1 cells. Complementation analysis demonstrated that mutants V.24.1 and HRP-1 were in the same complementation group. We also did complementation analysis with a third CHO cell mutant, DS28-6, reported in 1985 by Nakano and coworkers to be defective in secretion. DS28-6 cells were also in the same complementation group as V.24.1 and HRP-1. Thus, there are now three known members of the End4 complementation group. To biochemically analyze the defect in End4 mutants, we have used the semi-intact cell system described by Balch and coworkers to investigate transport between the endoplasmic reticulum and Golgi *in vitro*. Cytosol prepared at the restrictive temperature from mutants V.24.1, HRP-1 or DS28-6 supported transport from the endoplasmic reticulum to the Golgi using semi-intact CHO clone 15B cells, suggesting that the defect in the mutants is related to a membrane-associated protein. We are now attempting to reconstitute the mutant phenotype *in vitro* using semi-intact cells made with End4 mutants and cytosol from wild-type cells.

H 232 CHARACTERIZATION OF PHAGOCYTIC COMPARTMENTS IN J774 MACROPHAGES, Michel Desjardins, Lukas Huber, Robert Parton and Gareth Griffiths, EMBL, Heidelberg, Germany.

Internalization of latex beads in J774 macrophage leads to the formation of phagosomes. Only after their formation do phagosomes interact with organelles of the endocytic apparatus. Thus, when HRP, a marker of fluid phase endocytosis, is internalized to late endocytic compartments, subsequent internalization of latex beads lead to a progressive transfer of HRP to the phagosome, as showed by biochemical analysis and electron microscopy. Although the mechanism of this transfer is not known, video-microscopy shows that both phagosomes and late endocytic organelles are highly dynamic and make frequent contacts. Immunocytochemical localization of LAMP 1, a membrane protein of late endocytic organelles, at the electron microscope level showed the progressive acquisition of this protein by phagosomes with time. The phagocytic compartment was then isolated at different times after their formation by cell fractionation on a sucrose gradient. The analysis of phagosomes proteins metabolically labelled with ^{35}S methionine revealed a simple pattern on 2-D high resolution gel electrophoresis, when compared with the total membrane fraction. Using this assay, kinetic analysis of the formation of phagosomes showed the disappearance as well as the progressive appearance of distinct polypeptides after increasing times of latex uptake. Furthermore, GTP binding blots after 2-D gel electrophoresis showed the specific enrichment of several low molecular weight ras-like GTP-binding proteins, including rab 7, a late endosomal protein. These results suggest that phagosomes progressively acquire structural and functional properties of late endocytic organelles.

M.D. is the recipient of a post-doctoral fellowship from the Medical Research Council of Canada.

H 233 ENDOCYTOSIS OF *RHIZOBIUM* IN PLANT CELL AND PROLIFERATION OF ENDOMEMBRANES TO ENCLOSE THE INVADING BACTERIA, Choong-Ill Cheon, Na-Gyong Lee and D.P.S. Verma, Department of Molecular Genetics and Biotechnology Center, The Ohio State University, Columbus, OH 43210
The symbiotic interaction of *Rhizobium* and legume plant results in the development of specialized structures, root nodules, for nitrogen fixation. *Rhizobium* enters the plant cell through an endocytotic process, enclosed by peribacteroid membrane (PBM) derived from the host plasma membrane. Rapid proliferation of the endomembrane system occurs to accomodate more than 20,000 bacteroids per cell. We have initiated studies to search for plant genes involved in vectorial transport of proteins to PBM. YPT1, a small GTP-binding protein, mediates protein transfer from ER to Golgi in *Saccharomyces cerevisiae.* Since ultrastructure of the root nodules shows extensive vesicle formation and fusion events on the plasma membrane, infection thread membrane, and the PBM, YPT1 homolog(s) in legume may be important in forming PBM. A soybean homolog of YPT1(SOYPT1) was cloned and found to complement yeast *ypt1* mutant. Transgenic nodules containing antisense YPT1 under the leghemoglobin promoter (a nodule-specific promoter) were developed on hairy roots. These nodules showed retarded growth and reduced nitrogen fixation activity. We have also isolated five cDNAs encoding *ras*-related proteins from soybean and *Vigna.* The proteins are highly homologous among themselves(>90%) and contain all the conserved domains of vesicular-transport proteins. The expression of the YPT1 and *ras* homologs and their function in PBM biogenesis are under study.

H 234 ISOLATION OF TEMPERATURE-SENSITIVE ENDOCYTOSIS MUTANTS IN DICTYOSTELIUM DISCOIDEUM
Rebecca A. Bacon and Ira Mellman, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510
The identification of novel proteins involved in regulating endocytosis is critical for the further elucidation of endocytic membrane trafficking. Therefore, to define new gene products necessary for this process, we have taken a genetic approach using the haploid eucaryote, *Dictyostelium discoideum.* These cells are amenable to genetic manipulation and have a high capacity for both pinocytosis and receptor-mediated phagocytosis. Normal functioning of either process is necessary for growth. To isolate mutants, mutagenized cells were screened for an inability to internalize the fluorescent fluid-phase marker, FITC-Dextran, at the restrictive temperature using fluorescence-activated cell sorting. We have isolated 15 mutants, of which 9 have demonstrated a temperature-sensitive (ts) phenotype. All the mutants that show a ts defect in pinocytosis also have a ts growth defect.
In the tightest mutant identified, endocytosis is completely normal at the permissive temperature but is immediately reduced upon shift to the non-permissive temperature (26.5°C). By one hour, pinocytosis is blocked. In addition to being quickly induced, the phenotype is reversible. Recycling, degradation, and phagocytosis proceed normally at 26.5°C. These data suggest that the defect is occurring in an early step in the endocytic pathway. In addition, membrane localization of clathrin, secretion of a lysosomal enzyme, ATP levels, and contractile vacuole function are normal under non-permissive conditions. This mutant has a ts growth defect; no growth is seen in either liquid or bacterial co-cultures at the restrictive temperature. This mutant is a good candidate for complementation cloning due to this tight growth phenotype, a low reversion rate, and an high transformation frequency.
Other mutants demonstrate a slower appearance of the endocytic defect and a partial defect under permissive conditions. We are now carrying out complementation analysis on the mutants. The variety and specificity of phenotypes indicate that it is possible to specifically isolate endocytosis mutants in the organism *Dictyostelium discoideum.*

H 235 THE ROLE OF INVARIANT CHAIN CYTOPLASMIC TAIL IN CLASS II TRANSPORT. Lynne S Arneson, Beatrice Fineschi and Jim Miller. University of Chicago, Chicago, Il, 60637.

Invariant chain (Ii) complexes with class II MHC soon after synthesis and performs a number of functions. Ii has been shown to affect folding of class II in the ER and rate of transport to the Golgi, is thought to block class II binding of endogenous peptide in the ER, and has been shown to enhance presentation of some antigens. In addition, Ii is thought to affect class II transport. Studies have implicated the cytoplasmic tail of Ii as an endosomal localization signal for the class II/Ii complex. To determine whether this signal functions as a transport signal directing class II to the endosome, or as a retention signal delaying class II transit through the endosome, we are examining the effect of Ii on the biosynthetic transport route of class II. We have begun by characterizing the rate of class II arrival at the cell surface in the absence of Ii, the presence of wild type Ii, or the presence of a mutated form of Ii in which amino acids 2 through 17 have been removed from the cytoplasmic tail (ΔIi). We have found that class II is retained in the cell for a longer period when Ii is coexpressed. However, when ΔIi is present, class II/Ii complexes are transported to the surface at a rate similar to that seen in the absence of Ii. These data suggest that the cytoplasmic domain of Ii is responsible for the delay of class II transport from the Golgi to the surface. Our current experiments are directed at determining the mechanism by which Ii causes this effect on class II.

H 236 THE UPTAKE OF *BORRELIA BURGDORFERI* VIA COILING PHAGOCYTOSIS RESULTS IN NON-LYSOSOMAL DEGRADATION. Michael G. Rittig#, Thomas Häupl*, Michael Kressel§, Peter Groscurth§, and Gerd R. Burmester*. #Dept. of Anatomy and *Inst. of Clinical Immunology and Rheumatology, Dept. of Internal Medicine III, University of Erlangen-Nürnberg, Erlangen, Germany; §Dept. of Anatomy, Div. of Cell Biology, University of Zürich, Zürich, Switzerland.

Ultrastructural studies have shown that phagocytes internalize the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease, by two different uptake mechanisms. Conventional phagocytosis, which is the less frequent mechanism, leads to the regular lysosomal degradation of the spirochetes. On the contrary, the predominating mechanism of coiling phagocytosis results in an unusual intracellular pathway. To further analyze this pathway the processing of *B. burgdorferi* by human peripheral blood monocytes and neutrophilic granulocytes was investigated. Both electron and confocal laser-scan microscopy were used. With coiling phagocytosis a single pseudopod of the phagocyte contacted the *B. burgdorferi* cell. The pseudopod wrapped around the spirochete in multiple turns. Subsequently, the entire phagocytic complex was internalized. Disintegration of the coiled pseudopod membrane released the wrapped *B. burgdorferi* cell into the cytosol. However, the spirochete remained within the pseudopod-derived cytoplasm, tightly enclosed by the microfilament network of the former pseudopod. No cellular organelles such as lysosomes were seen within these distinct cytosolic areas. Finally, the spirochete was degraded without the apparent participation of lysosomes. In conclusion, coiling phagocytosis of *B. burgdorferi* results in a degradation mechanism which is not usually observed for the uptake of bacteria. These microbes do not disintegrate in phagolysosomes but in distinct, organelle-free cytosolic areas, probably by the aid of non-lysosomal catabolic enzymes. This unusual processing may be important for the presentation of bacterial antigens in the context of the MHC class I antigens. Therefore, studies using the model of *B. burgdorferi* will provide new insights in processing and presentation of bacterial antigens.

H 237 DESIGN OF *de novo* TARGETING PEPTIDES FOR ENDOCYTOSIS AND NUCLEAR TRANSLOCATION OF DRUGS, Jean Gariépy, Jim Ferguson and Katherine Sheldon, Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9.

In an effort to develop agents that can selectively target cell compartments, we have designed novel peptides that code for three functional domains active in live cells. The three domains include a cell translocation domain allowing the peptides to cross the cytoplasmic membrane of live cells, a nuclear localization domain that leads to the accumulation of the peptides in the nucleus of such cells and, a DNA intercalating moiety that traps the peptides in the nucleus. These peptides are spontaneously taken up by cells and are cytotoxic to cells at concentrations greater than 10 μM . However, one can readily monitor the internalization of these peptides at concentrations near 1 μM where all cells are viable. The uptake of peptides incorporating the DNA intercalator acridine was directly followed by performing fluorescent microscopy on live cells. The peptides were also radiolabeled with iodine-125 to analyze their uptake by cells. Our results indicate that in relation to the complete prototype peptide, a peptide that lacks the cytoplasmic translocation domain penetrates live cells poorly. In contrast, the nucleus and cytoplasm of cells remain evenly fluorescent when exposed to a truncated form of the peptide lacking the nuclear localization domain. The peptide containing all three domains rapidly accumulates in the nucleus of cells. The phenomena was observed in several unrelated cell lines (CHO, MCF-7, and Vero cells) and demonstrates that one can design peptide-based agents that are taken up by live cells and rapidly sorted into compartments such as the nucleus. (Supported by NCI of Canada)

H 238 DIFFERENTIAL ER RETENTION AND PROCESSING OF ALPHA AND BETA-SUBUNITS OF P-TYPE ATPases IN XENOPUS OOCYTES, Käthi Geering, Philippe Jaunin and Ahmed Beggah, Institut de Pharmacologie et Toxicologie de l'Université, rue du Bugnon 27, CH-1005 Lausanne, Switzerland

Among the family of P-type ATPases (characterized by the formation of an aspartyl phosphate intermediate during ATP hydrolysis), Na,K-ATPase (NKA) and H,K-ATPase (HKA) are the only members that are composed of two subunits. The alpha-subunits carry all catalytically relevant domains but the beta-subunits are important for the structural and functional maturation of the enzymes. In Xenopus oocytes, alpha-subunits of endogenous NKA are synthesized in excess over beta-subunits. The unassembled alpha-proteins are retained in the ER, have a long half-life and maintain the ability to associate with exogenous NKA beta-subunits over a prolonged time. On the contrary, exogenous Xenopus NKA alpha-subunits synthesized in the absence of beta-subunits are rapidly degraded in the ER. Coexpression of NKA or HKA beta-subunits stabilizes the exogenous NKA alpha-subunits and permits their exit from the ER. Xenopus NKA beta1- and beta3-isoforms synthesized from injected cRNA also need oligomerization with alpha-subunits to leave the ER while rabbit HKA beta-subunits are transported to distal Golgi compartments and become fully glycosylated even in the absence of concomitant synthesis of alpha-subunits. Together these data show that proteins of high homology are processed in different ways in Xenopus oocytes and indicate that the ER retention and degradation system in these cells is highly selective. To characterize the structural domains in the NKA beta-subunit which mediates ER retention we prepared chimeric proteins between NKA and HKA beta-subunits as well as between transferrin receptors and NKA and HKA beta-subunits respectively. Our results indicate that the structural integrity of both the N-terminal transmembrane and the C-terminal extracytoplasmic domain of Xenopus NKA beta-subunits is needed to promote ER retention of individual subunits in Xenopus oocytes.

H 239 COMPARTMENTALIZATION AND SIGNAL TRANSDUCTION: I. BASOLATERAL-APICAL DISTRIBUTION OF INOSITOL 1,4,5-TRISPHOSPHATE 5-PHOSPHATASE IN EPITHELIAL CELLS. Stephen B. Shears and W. Howard Evans. Laboratory of Cellular and Molecular Pharmacology, NIEHS, NIH, Research Triangle Park, NC 27709.

The binding of extracellular agonists to the basolateral surface of epithelial cells causes the intracellular release of Ins(1,4,5)P_3, a near-ubiquitous signal that mobilizes Ca^{2+} stores, thereby controlling the activity of many Ca^{2+}-dependent enzymes. Metabolism of the trisphosphate by a 3-kinase and a 5-phosphatase comprise signalling off-switches. The 3-kinase is largely soluble, but in most non-hemopoietic cells the 5-phosphatase predominates in the plasma membrane. In hepatocyte basolateral (sinusoidal) and apical (canalicular) plasma membranes the specific activity of the 5-phosphatase (nmol/mg protein/min) was 35 ± 2 and 140 ± 5 respectively (n=4). Thus the sites of generation and hydrolysis of Ins(1,4,5)P_3 are compartmentalized at opposite poles of the cell, perhaps generating intracellular concentration gradients of second messengers. The 5-phosphatase appears to be delivered to the apical membranes inside endosomes; the specific activity inside 'early' and 'late' endosomes was 22 ± 5 and 71 ± 9 (n=4) respectively. In intestinal epithelial cells the 5-phosphatase specific activities in basolateral and apical plasma membranes were 114 ± 10 and 18 ± 3 respectively (n=4). Thus, in intestinal epithelial cells the sites of generation and inactivation of Ins(1,4,5)P_3 appear to be proximal. The differential polarized intracellular distribution of components of signal transduction in epithelial cells suggests cell dependent variation in gradients of intracellular signals that may contribute to cell-specific responses.

H 240 DETECTION OF OLIGOMERIC FORMS OF P-GLYCOPROTEIN IN MULTI-DRUG RESISTANT (MDR) MAMMALIAN CELL MEMBRANES. Marianne S. Poruchynsky and Victor Ling, Division of Molecular and Structural Biology, The Ontario Cancer Institute, Toronto, Ontario, Canada, M4X-1K9.

P-glycoprotein (P-gp) is expressed in a variety of normal tissues and is often found in high levels in tumors which fail to respond to chemotherapeutic drugs. P-gp is believed to function as a drug efflux pump at the plasma membrane, but the functional form of P-gp in its native state is unknown. In the present study, cell lines which exhibit the MDR phenotype and express P-gp, were radiolabeled and extracted with nonionic detergent CHAPS. When the postnuclear lysate was subjected to sucrose gradient velocity sedimentation, and P-gp detected with specific antibodies, the sedimentation profile indicated that >50% of the P-gp sedimented as higher order oligomers. Oligomers were preserved, although to a much lesser extent, when detergents TX100 or NP40 were used. In contrast, when SDS was used to solublize cells, monomers were exclusively retrieved and some of the naturally occurring oligomers could only be preserved if the crosslinker dithiobis-succinimydyl-proprionate (DSP) was used prior to SDS solublization. Oligomerization appears to take place in an early biosynthetic compartment for both the unprocessed and the early processed forms of P-gp. Moreover, when cells were treated with Brefeldin A, the sedimentation pattern for all forms of P-gp was the same as that of untreated cells. To determine if the oligomeric state of P-gp was functionally different from the monomeric, we examined binding of a photoactive analog of ATP (azido-ATP) using a total membrane preparation which was UV crosslinked prior to CHAPS solublization. It appeared that both forms bind ATP with equal efficiency. Similar experiments are underway to determine the in vitro binding of ^{3}H-azidopine, a P-gp substrate. P-gp is a phosphoprotein and the state of phosphorylation may be important for P-gp function. By in vivo labeling with ^{32}P-ortho-phosphate, we observed that the monomer was more highly phosphorylated than the oligomers. Thus, the oligomers may be functionally distinct from the monomers. We conclude that oligomers of P-gp exist in the plasma membrane of MDR cells and form via non-covalent interactions. We speculate that the dynamics of their formation/dissociation may be important in the mechanism of action of P-gp.This work is supported by NCI of Canada and the USA.

H 241 THE HIV-1 Vpu PROTEIN INDUCES RAPID DEGRADATION OF CD4.

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CD4 is an integral membrane glycoprotein which is known as the HIV receptor for infection of human cells. The protein is synthesized in the endoplasmic reticulum (ER) and subsequently transported to the cell surface via the Golgi complex. HIV-infection of $CD4^+$ cells leads to downmodulation of cell surface CD4, due at least in part to the formation of stable intracellular complexes between CD4 and the HIV-1 Env precursor polyprotein, gp160. This process "traps" both proteins in the ER leading to reduced surface expression of CD4 and results in reduced processing of gp160 to gp120 and gp41. We have recently demonstrated that the presence of the HIV-1 encoded integral membrane protein, Vpu, can reduce the formation of Env/CD4 complexes resulting in increased gp160 processing and decreased CD4 stability. We have studied the effect of Vpu on CD4 stability and found that Vpu induces rapid degradation of CD4, reducing the half-life of CD4 from 6 hours to 12 minutes. By using a CD4 binding mutant of gp160 we were able to show that this Vpu-induced degradation of CD4 requires retention of CD4 in the ER which is normally accomplished through its binding to gp160. The involvement of gp160 in the induction of CD4 degradation is restricted to its function as a CD4 trap since in the absence of Env an ER retention mutant of CD4, or wild type CD4 in cultures treated with brefeldin A, a drug that blocks transport of proteins from the ER, are degraded in the presence of Vpu.

H 242 THE UROKINASE PLASMINOGEN ACTIVATOR PATHWAY AS A NOVEL MECHANISM OF TUMOR TARGETING AND CELL MEMBRANE TRAVERSAL. Jerzy Jankun, Department of Chemistry, The University of Toledo, Toledo, OH 43606-3390.

In order to metastasize the malignant cell must degrade the adjacent connective tissue. Since this type of tissue contains mainly proteins, it has been postulated that the proteolytic enzymes are involved in the process of tumor cell migration. One of them is the urokinase plasminogen activator (uPA) synthesized by many human malignancies. The uPA can be bound to the cell surface via its receptor (uPAR). The uPA/uPAR complex stay on the cell surface for a prolonged period of time. The receptor bound uPA is vulnerable to inhibition by plasminogen activator inhibitor type-1 (PAI-1). Upon binding the PAI-1 to the uPA/uPAR complex, the inhibitor triggers the series of events leading to the internalization of whole PAI-1/uPA/uPAR complex via endocytosis. This property of PAI-1 was utilized to develop the cancer cell localizing and internalizing cytotoxic conjugate. The PAI-1 and A-chain of cholera toxin have been noncovalently bound to the colloidal gold nanoparticles. The A-chain of cholera toxin can not be endocytosed itself. This function was preformed by PAI-1 molecule. The HT1080, fibrosarcoma derived cell line, expressing high amount of the receptor-bound uPA, and KD, fibroblastic cell line, expressing low amount of receptor-bound uPA were treated with colloidal gold nanoparticles bearing the PAI-1 and A-chain cholera toxin. As a negative control only the A-chain cholera toxin bound to the gold was used. The second negative control included the cells where the receptor-bound uPA was saturated with high amount of PAI-1 (est. 50 times more that uPA). As expected, the treatment with PAI-1/A-chain cholera toxin/colloidal gold complex killed more cells expressing high amount of receptor-bound uPA than cells expressing low amount of this enzyme. No cell killing was observed for negative controls.

H 243 COMPARTMENTALIZATION AND SIGNAL TRANSDUCTION. II. HEPATIC INOSITOL 1,3,4,5 TETRAKISPHOSPHATE 3-PHOSPHATASE IS SEQUESTERED INSIDE ENDOPLASMIC RETICULUM. Nawab Ali, Andrew Craxton and Stephen B. Shears. Laboratory of Cellular and Molecular Pharmacology, NIH, NIEHS, Research Triangle Park, NC 27709.

Inositol-1,4,5-trisphosphate, the Ca^{2+}-mobilizing intracellular signal, can be removed from the cell by a 3-kinase. By countering this metabolism, inositol-1,3,4,5-tetrakisphosphate 3-phosphatase may help sustain Ca^{2+} signalling during cell activation, but controversy surrounds this proposal. Investigations into the subcellular location of the 3-phosphatase would help elucidate its function, but such work is seriously compromised by the presence of potent heat-stable inhibitor(s) of the enzyme (M.E. Hodgson and S.B. Shears, 1990 *Biochem. J.* **267** 831-834). Separation of the inhibitor from the enzyme by anion-exchange chromatography indicated that no more than 5-10% of cellular activity was cytosolic. Upon gel filtration the inhibitor co-eluted with inositol hexakisphosphate (a high-affinity competing substrate of the 3-phosphatase, Nogimori *et al.*, (1991) *J. Biol. Chem.* **266** 16499-16506). Subcellular fractions were pre-treated with phytase to deplete endogenous inhibitor, and then 90-95% of hepatic 3-phosphatase was found to be restricted to the endoplasmic reticulum, with little or no activity in either plasma membranes, mitochondria or nucleii. Treatment of microsomes with either CHAPS or Triton X-100 revealed the latency of 3-phosphatase (93 ± 8%, n=7) to be identical to that of mannose 6-phosphatase (93 ± 2%, n=12), an intraorganelle marker. Treatment of microsomes with sodium carbonate or phosphatidylcholine-specific phospholipase C, to specifically release luminal contents, solubilized the 3-phosphatase activity. Thus, hepatic 3-phosphatase appears to have a highly restricted access to inositol polyphosphates *in vivo* that suggests the enzyme has an alternative primary role.

H 244 CLONING OF AN APICAL PROTEIN FROM *XENOPUS LAEVIS* THAT PARTICIPATES IN AMILORIDE-SENSITIVE SODIUM CHANNEL ACTIVITY. Olivier Staub, Bernard C. Rossier, Jean-Pierre Kraehenbuhl. Institut de Biochimie and ISREC, CH-1066 Epalinges, Institut de Pharmacologie, CH-1005 Lausanne, Switzerland.

Amiloride-sensitive sodium channels are present on the apical membrane of high resistance, sodium transporting epithelia, including A6 cells derived from *Xenopus* kidney. We have isolated a cDNA encoding a 1420 amino acid long polypeptide with no signal sequence, a putative transmembrane segment, and 3 predicted amphipathic α helices. An antibody raised against a fusion protein containing C terminal parts of the protein and an anti-idiotypic antibody known to recognize the amiloride-binding site of the epithelial sodium channel [Kleyman et al. (1991) J. Biol. Chem. **266**: 3907-3915] immunoprecipitated a similar protein complex from $[^{35}S]$methionine-labeled and from apically-radioiodinated A6 cells. A single ~130 kDa protein was recovered from samples reduced with dithiothreitol. The antibody also crossreacted by ELISA with the putative amiloride-sensitive sodium channel isolated from A6 cells [Benos et al. (1987) J.Biol.Chem. **262**: 10613-10618]. Complementary RNA injected into oocytes did not reconstitute amiloride-sensitive sodium transport, but antisense RNA or antisense oligodeoxynucleotides specific for two distinct sequences of the cloned cDNA inhibited amiloride-sensitive sodium current induced by injection of A6 cell mRNA. We propose that the cDNA encodes an apical plasma membrane protein that plays a role in the functional expression of the amiloride-sensitive epithelial sodium channel. It may represent either a subunit of the *Xenopus laevis* sodium channel or a regulatory protein essential for sodium channel function.

Clathrin, the Cytoskeleton and Membrane Traffic in Yeast Cells

H 301 INSULIN RESPONSE AND THE CYTOSKELETON IN MUSCLE CELLS, POSSIBLE RELATIONSHIPS, T. Tsakiridis, T. Ramlal, Y. Mitsumoto, M. Vranic and A. Klip. Dept. of Cell Biology, The Hospital for Sick Children, M5G 1X8, and Dept. of Physiology, University of Toronto, M5S 1A8, Toronto, Ontario, Canada.
In muscle cells glucose transporters present in intracellular vesicles are thought to be recruited to the plasma membrane in response to acute insulin treatment. We have studied the L6 muscle cell line which differentiates in vitro from myoblasts to myotubes and expresses the glucose transporter isoforms GLUT 1, 3 and 4. Subcellular fractionation studies have indicated that all three isoforms translocate from an intracellular light membrane compartment (LM) to the plasma membrane (PM) of L6 cells in response to insulin. Here we report that isolated PM from L6 cells contain high, and LM lower, levels of clathrin. Immunofluorescence studies showed labelling of the cell periphery and of filament-like structures. The components of the cytoskeleton were investigated in fixed cells and in membrane fractions. The actin network, revealed by rhodamine-labelled phalloidin, was detected as longitudinal thick filaments in L6 myoblasts, which became thinner upon cell differentiation. Cytochalasin D (1 μM) disrupted the actin network, causing dramatic but rapidly reversible changes in cell shape. The microtubule network, revealed by immunofluorescence developed from an area close to the nucleus (MTOC) in myoblasts, while in myotubes it grew out of nuclei aggregates. Colchicine (1μM) disrupted the microtubule network without any effect on cell morphology. The microtubule-associated mechanoenzyme kinesin was detected in L6 cells by immunofluorescence studies and Western blotting revealed immunoreactive bands of about 110kDa in PM and LM. Acute insulin treatment caused aggregation of actin filaments in L6 myotubes. Aggregation of immunofluorescence was also apparent for kinesin and glucose transporter labelling following insulin treatment. These results indicate that L6 cells are a suitable system to study the effects of insulin on glucose transporter distribution and its possible mediation by cytoskeletal components.

H 302 β- AND γ-CENTRACTIN: TWO NEW MEMBERS OF THE CENTRACTIN FAMILY OF ACTIN-LIKE PROTEINS, Sean W. Clark and David I. Meyer, Department of Biological Chemistry and the Molecular Biology Institute, University of California, Los Angeles, CA 90024
We recently identified a novel actin homologue, centractin. By biochemical and morphological criteria, centractin is localized in centrosomes (Clark and Meyer (1992) *Nature* 359:246-250) and the p150^{glued}/dynactin complex (Holzbaur *et al.* (1990) *Nature* 351:579-583; Lees-Miller *et al.* (1992) *Nature* 359:244-246). Using high stringency Northern blotting, we have identified two new members of the centractin family. The larger of these messages has been cloned, sequenced, and is clearly an isoform of the original α-centractin. We have tentatively named this isoform β-centractin. α- and β-centractin share more than 90% amino acid identity and greater than 95% similarity. As with α-centractin, β-centractin has consensus amino acid motifs known to interact with ATP and divalent cations in actin. Both β- and γ-centractin are encoded by smaller mRNA species than α-centractin due to a shorter 3' untranslated region, in the case of β-centractin. Studies using anti-centractin antibodies and 2-D electrophoresis are underway to determine the stoichiometry and localization of these three isoforms.

H 303 BREFELDIN A-INDUCED RELOCATION OF α-MANNOSIDASE IS DEPENDENT ON MICROTUBULE FUNCTION. Quoc V. Nguyen, Allysen M. Roskey, Department of Pediatrics, SUNY Health Science Center, Syracuse, NY 13210

We have characterized the structural changes in class II major histocompatibility complex (MHC) and its associated invariant chain (Ii) during processing and intracellular transport in the presence of the antibiotic brefeldin A (BFA) and the tubulin-disrupting agent nocodazole (ND). Treatment with ND was not associated with any change in processing or time course of transport of MHC class II to cell surface. Transport through the golgi was blocked by BFA treatment, which also increased the half life of class II-associated Ii. BFA treatment was associated with gradual decrease in molecular weights (MW) of immunoprecipitated class II and Ii by approximately 2kD as a function of time. The incubation with both ND and BFA was associated with a further 2kD decrease in MW of class II-Ii complex as compared to BFA treatment alone. The MW lowering effect of chased molecules could be generated in vitro by α-mannosidase treatment. The use of either 1-deoxymannojirimicin or swainsonine blocked the MW lowering effect of combined BFA and ND treatment. These observations suggested that the relocation of newly synthesized class II-Ii complex to a salvage compartment by BFA exposed them to trimming glycosidase activity. This decrease in MW was augmented by ND treatment, probably due to a decrease in enzyme degradation which could be dependent on microtubule function.

H 304 SUPPRESSORS OF CLATHRIN DEFICIENCY: OVER-EXPRESSION OF UBIQUITIN RESCUES THE LETHALITY OF CLATHRIN-DEFICIENT YEAST, K. Nelson, M. Holmer, J. Weigle and S.K. Lemmon, Dept. of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106.
Clathrin-mediated vesicular transport is important for normal growth of yeast. Previously, we identified a genetic locus (*SCD1*) that influences the ability of clathrin heavy chain-deficient (Chc$^-$) yeast to survive. With the *scd1-v* allele Chc$^-$ yeast are viable but grow poorly; with the *scd1-i* allele Chc$^-$ cells are inviable. In order to identify the *SCD1* locus and other genes that can rescue the inviability of *chc1-Δ scd1-i* yeast, a multicopy suppressor selection strategy was developed. A strain of *scd1-i* genotype carrying the clathrin heavy chain gene under *GAL1* control (*GAL1:CHC1*) was transformed with a YEp24 yeast genomic library and colonies that could grow on glucose were selected. Plasmids from six distinct genetic loci (*SCD2-SCD7*) were recovered. Here we present our analysis of *SCD2*, *SCD3* and *SCD7*.

SCD7 only rescues in high copy and is not allelic to *SCD1*. *SCD7* encodes a polypeptide with no significant homology to known proteins; however, it is an essential gene in *CHC1* strains. *SCD3*, originally recovered as a multicopy suppressor of clathrin deficiency, was also isolated from a centromere library as a single copy suppressor. Therefore, it is a good candidate for *SCD1*. Integrative transformation and segregation analysis are being performed to determine whether *SCD1* and *SCD3* are allelic, and the complementing region is being narrowed for sequence analysis.

SCD2 rescues only in high copy and is identical to *UBI4*, the polyubiquitin gene. The conjugation of ubiquitin to intracellular proteins can mediate their selective degradation. Since *UBI4* is required for survival of yeast cells undergoing stress and is induced during starvation, we examined ubiquitin expression in *GAL1:CHC1* yeast. After shift from galactose to glucose medium to repress synthesis of clathrin heavy chains, *UBI4* mRNA levels were elevated >10 fold while the quantity of free ubiquitin declined several fold relative to that of Chc$^+$ cells. In addition, novel higher molecular weight ubiquitin conjugates appeared in clathrin-deficient yeast. We suggest that higher levels of ubiquitin are required for turnover of mislocalized or improperly processed proteins that accumulate in the absence of clathrin and that ubiquitin may play a general role in turnover of proteins in the secretory and/or endocytic pathways.

H 305 *Abstract Withdrawn*

H 306 *Abstract Withdrawn*

H 307 CHARACTERIZATION OF YEAST SMALL CLATHRIN ASSOCIATED PROTEINS. H. L. Phan, J. A. Finlay, G. S. Payne, Molecular Biology Institute and Dept. of Biological Chemistry, UCLA, Los Angeles, CA 90024.

Clathrin-coated vesicles (CCVs) in mammals consist of clathrin heavy and light chains and clathrin associated proteins (APs). Distinct, but homologous complexes of APs are differentially localized in mammalian cells, one is found at the plasma membrane and one is found at the Golgi complex. Database searches with the predicted sequences of mammalian AP subunits have revealed a number of genes encoding*S. cerevisiae* homologues, including a 17 kDa small associated protein (APS) homologue (APS1). APS1 encodes a protein (Aps1p) which displays 50% amino acid sequence identity with its counterpart at the mammalian plasma membrane. We have isolated another APS homologue (APS2) in *S. cerevisiae* using the polymerase chain reaction with degenerate oligonucleotides primers derived from conserved sequences of APSs. Aps2p is most identical (52%) to the mammalian Golgi APSs.

Antibodies specific for Aps1p and Aps2p have been used in fractionation studies. Aps1p and Aps2p are found both in the cytosol and on membranes. The membrane bound Aps1p and Aps2p can be removed by treatment with 0.5M Tris HCl (pH7.0). Sizing chromatography indicates that both Aps1p and Aps2p exist in complexes >200 kDa in size. These biochemical features of Aps1p and Aps2p are consistent with those reported for mammalian APs.

Gene disruption of APS1 and APS2 either individually or together, yields strains with no discernable abnormalities.However, the aps mutations accentuate abnormal phenotypes caused by a temperature sensitive allele of clathrin heavy chain (chc1-ts). At 24°C, MATα cells harboring the chc1-ts allele secrete mature a-factor, whereas at 37°C, the majority of secreted α-factor is not proteolytically matured. Disruptions of APS1 and/or APS2 in chc1-ts yeast strains accentuates this α-factor processing defect, such that α-factor precursor is secreted 24°C. This accentuation is most pronounced in chc1-ts, aps1Δ, aps2Δ strains. In chc1-ts cells carrying single apsΔ mutations, aps2Δ causes amore severe maturation defect than aps1Δ. Also the maturation defect reaches a maximum in the multiply mutant strains at 30°C instead of 37°C. Preliminary studies indicate that Kex2p may also be mislocalized to the cell surface in these strains at 24°C. In addition, the growth of chc1-ts strains at 37°C is further compromised when APS2 is disrupted (but not when APS1 is disrupted). We are currently investigating the role of APS1 and APS2 in other aspects of intracellular protein traffic.

H 308 *DROSOPHILA* CLATHRIN HEAVY CHAIN GENE: CLATHRIN FUNCTION IS ESSENTIAL IN A MULTICELLULAR ORGANISM, S.K. Lemmon, A.L. Katzen, M. Morgan, A.P. Mahowald, and C. Bazinet, Dept. of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland OH, 44106, George Williams Hooper Foundation, University of California, San Francisco, CA 94143, Dept. of Molecular Genetics and Cell Biology, University of Chicago, 920 E. 58th St., Chicago IL 60637.

The identification of genes encoding clathrin and associated proteins in a complex metazoan makes possible the genetic analysis of clathrin functions in the specialized cell types of differentiated tissues and organs. We have identified a genetic complementation group encoding the clathrin heavy chain (Chc) of *Drosophila melanogaster* by germline transformation with a modified P transposable element carrying the cloned *Chc* gene. The P[*Chc*] element complements a group of four mutations mapping to the *Chc* locus on the *X* chromosome. Three of these mutations are lethal, blocking development late in embryogenesis. Development probably proceeds this far due to the presence of substantial quantities of maternal clathrin transported into the oocyte from heterozygous nurse cells. A fourth allele, Chc^4, is leaky in the sense that Chc^4 hemizygous males survive to adulthood at low but significant frequencies. These individuals appear morphologically and behaviorally normal but are sterile. Examination of their testes reveals a defect in sperm motility and the presence at many sites on individualized sperm of a widening or dilation of the cell membrane. The defects are completely rescued by a wild-type copy of the *Chc* gene, arguing that they are due to the *Chc* mutation and not some other mutation in the genetic background of flies we are examining. These observations suggest a role for clathrin in the individualization of sperm, during which spermatids become invested in their own cell membrane after having developed in a syncitial cyst cell. Sperm individualization is an extraordinarily active, coordinated process of membrane morphogenesis and reorganization likely to be especially sensitive to defects in cellular transport.

H 309 ANALYSIS OF HSC70 INTERACTION WITH CLATHRIN FROM CELLS DEFICIENT IN LIGHT CHAIN A.
Darren H. Wong[1], Susan L. Acton[1], Peter Parham[2] and Antony P. Jackson[3], Frances M. Brodsky[1]; [1]Departments of Pharmacy and Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446, [2]Department of Cell Biology, Stanford University, CA 94305, and [3]Department of Biochemistry, University of Cambridge, CB2 1QW England.

Hsc70 is an ATPase that catalyzes the *in vitro* removal of clathrin triskelia from clathrin-coated vesicles (CCV). This uncoating process requires ATP hydrolysis as well as the presence of clathrin light chains on triskelia. Of the two light chains found in mammalian clathrin, LCa is more efficient than LCb in stimulating hsc70 ATPase activity and uncoating *in vitro*. To address the possible *in vivo* role of hsc70, we are currently studying various aspects of CCV-hsc70 interactions in A35 cells, an LCa-deficient clone of PC12 cells.

When hsc70 is incubated with crude CCV derived from A35 and wild type control cells in the presence of apyrase-hydrolyzed ATP, less hsc70 sediments at 100,000 x g with A35 CCV as compared to wild type CCV. This result suggested that uncoating may be less efficient in the LCa-deficient cell line. Two other experimental strategies to address this question are currently underway. One set of experiments compares the uncoating rate of CCV in A35 and wild type cells. Crude CCV are isolated from both cell lines and subjected to an *in vitro* uncoating reaction with hsc70 purified from bovine brain. The uncoating rate is then determined by measuring the amount of clathrin released after various timepoints. Preliminary results suggest the uncoating rate is slowed in A35 cells. The second experimental approach examines the time endocytosed ligand is associated with CCV in A35 and wild type cells. Cells will be allowed to internalize labeled ligand for various times. CCV will be immunoisolated from ruptured cells and assayed for the labeled ligand. If clathrin uncoating is less efficient in A35 cells, labeled ligand will remain associated with CCV for a longer period of time as compared to wild type cells. These results should provide evidence for or against the role of hsc70 in CCV uncoating *in vivo*.

H 310 THE LATERAL MOBILITY OF THE H1 HUMAN ASGP RECEPTOR IS AFFECTED BY INTERACTIONS WITH COATED PITS, Yoav I. Henis[1], Nurit Nardi[1], Ziva Katzir[1], Iris Geffen[2] and Christian Fuhrer[2], [1]Department of Biochemistry, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel; [2]Department of Biochemistry, University of Basel, Switzerland.
We have recently used lateral mobility studies to demonstrate dynamic interactions between a mutant influenza hemagglutinin and coated pits (Fire, E., D. E. Zwart, M. G. Roth, and Y. I. Henis. 1991. J. Cell Biol. 115:1585-1594). To investigate such interactions in natural membrane receptors, we have now studied H1, the major subunit of the human asialoglycoprotein receptor. The lateral mobility of H1 was measured by fluorescence photobleaching recovery (FPR) in live cells expressing H1 and H2 (HepG2 human hepatoma cells, and 1-7-1, a doubly transfected NIH3T3 line), or in stably transfected NIH3T3 cells singly expressing H1 (1-7 cells) or a mutant H1 in which the single cytoplasmic tyrosine (position 5) was replaced by alanine (5A-H1; F1(5A)-1 cells). The lateral diffusion coefficient (D) of 5A-H1 was 2-fold higher than that of H1 (whether expressed singly or together with H2), as expected if the interactions of 5A-H1 with coated pits were weaker. To investigate this point, we studied the effects of treatments that either disperse clathrin lattices (incubation in hypertonic medium) or alter their structure (cytosol acidification). Hypertonic medium treatment raised D of H1 and 5A-H1 to the same final level, without affecting their mobile fractions. This demonstrates that the lateral motion of H1 and, to a lesser extent, of 5A-H1, is inhibited by dynamic interactions with coated pit structures. Acidification of the cytoplasm, which retains altered clathrin lattices attached to the membrane, immobilized part of the H1 molecules, presumably due to entrapment in coated pits for the entire duration of the FPR measurement. Interestingly, the lateral mobility of 5A-H1 was not affected by cytosol acidification. Thus, it is possible that the enhanced interaction of H1 with coated pits following cytosol acidification involves mainly the tyrosine signal missing in 5A-H1.

H 311 RECONSTITUTION OF CLATHRIN-COATED PIT NUCLEATION, William G. Mallet, Michael P. Chang, and Frances M. Brodsky, Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143
Clathrin-coated pits are principally composed of two multi-subunit proteins, clathrin and adaptors. Adaptors bind directly to the plasma membrane and interact with the cytoplasmic domains of receptors. Clathrin forms a polymerized lattice surrounding the adaptors, mediating sequestration and internalization of associated receptors. We have established an in vitro binding assay to analyze the association of adaptors and clathrin with plasma membrane fragments that remain bound to tissue culture wells following freeze-thaw lysis of cells. Exposure of membrane fragments to high ionic strength buffer (0.5M Tris) removes clathrin-coated pit coat proteins. Purified exogenous adaptors then can bind to the "stripped" membranes in a saturable and high-affinity manner. However, purified clathrin does not subsequently bind to the membranes. We are investigating whether coat protein binding is affected by other factors present in cytosol or on the plasma membrane. Adaptors that are incubated with cytosol at 37°C prior to addition to stripped membranes show enhanced binding to the membranes, compared with untreated adaptors. This enhancement is not observed if cytosol is left out of the 37°C incubation. Enhanced adaptor binding suggests that some modifying reaction or component(s) in the cytosol influences coated pit nucleation. However, pre-incubation of coat proteins with cytosol does not promote the association of clathrin with Tris-treated membranes. Rather, high-affinity binding of clathrin to "stripped" membranes requires additional membrane-bound factor(s) which are apparently released upon Tris buffer treatment. Therefore, clathrin-coated pit nucleation is influenced by both peripheral membrane and cytoplasmic components.

H 312 CLATHRIN ASSEMBLY PROTEIN AP180: PRIMARY STRUCTURE, DOMAIN ORGANIZATION AND IDENTIFICATION OF A CLATHRIN BINDING SITE.
S. A. Morris[1], S. Schröder[1], U. Pleßmann[2], K. Weber[2] and E. Ungewickell[1]*
1)Max-Planck Institute for Biochemisty, D-8033 Martinsried, Germany
2Max-Planck Institute for Biophysical Chemistry, Dept. of Biochemistry, D-3400 Göttingen, Germany

Binding of AP180 to clathrin triskelia induces their assembly into 60-70 nm coats. We have isolated and sequenced cDNAs coding for rat AP180. The largest rat brain cDNA clone isolated predicts a molecular weight of 91,430 daltons for AP180. Two cDNA clones have an additional small 57 base pair insert. The deduced molecular weight agrees with gel filtration results when the more chaotropic denaturant 6 M guanidinium thiocyanate is used instead of the weaker guanidinium chloride. The sequence and the proteolytic cleavage pattern suggest a three domain structure. The N-terminal 300 residues (pI 8.7) harbor a clathrin binding site. An acidic middle domain (pI 3.6, 450 residues), interrupted by an uncharged alanine rich segment of 59 residues, seems responsible for anomalous physical properties of AP180. The C-terminal domain (166 residues) has a pI of 10.4. AP180 mRNA is restricted to neuronal tissues. AP180 shows no significant homology to known clathrin binding proteins but is nearly identical to a mouse phosphoprotein (F1-20). This protein, localized to synaptic termini, has so far been of unknown function. We propose that AP180(f1-20) has a neuron specific function in clathrin-coated vesicle assembly.

H 313 RECONSTITUTION OF A MAMMALIAN AP-2 COMPLEX FROM ITS RECOMBINANT SUBUNITS EXPRESSED IN E. coli, Andreas Gallusser and Tom Kirchhausen, Harvard Medical School/CBR, Boston, MA 02115
With the goal of reconstituting functional clathrin-associated protein complexes (APs or adaptors), we have overexpressed in E. coli α_c, β, AP50 and AP17, the subunits of the plasma membrane AP-2 complex. This approach facilitates the analysis of subunit interactions within the complex and allows to study AP functions by site-directed mutagenesis. The recombinant subunits were partially purified as inclusion bodies and subjected to complete denaturation in 6M guanidine hydrochloride. They were then refolded, either alone or in combination with each other by dialysis at protein concentrations in the range of 20-100μg/ml. The refolded β chain was completely soluble and limited tryptic digestion was consistent with its reconstitution into a two-domain structure similar to the β chain in native AP-2 complexes. Direct interaction between the refolded β chain and clathrin was shown by its efficient binding to preformed clathrin cages in the nanomolar range. Upon their simultaneous refolding, β and AP17 interact with each other with the same 1:1 molar stoichiometry determined in native AP-2 complexes. In contrast, AP50 binds poorly to the β chain. Studies are underway to demonstrate that recombinant AP-2 complexes can drive clathrin coat assembly in the same way as native AP-2 complexes.

H 314 CLATHRIN ASSEMBLY PROTEIN AP180: PRIMARY STRUCTURE, DOMAIN ORGANIZATION AND IDENTIFICATION OF A CLATHRIN BINDING SITE, Ernst Ungewickell[1], Stephen A. Morris[1], Stephan Schröder[1], Uwe Plessmann[2] and Klaus Weber[2]. Max-Planck Institute for Biochemistry, D-8033 Martinsried (1) and Max-Planck Institute for Biophysical Chemistry, Dept. of Biochemistry, D-3400 Göttingen, Germany (2).
Binding of AP180 to clathrin triskelia induces their assembly into 60-70 nm coats. The largest rat brain cDNA clone isolated predicts a molecular weight of 91,430 for AP180. Two cDNA clones have an additional small 57 bp insert. The deduced molecular weight agrees with gel filtration results provided the more chaotropic denaturant 6 M guanidinium thiocyanate is substituted for the weaker guanidinium chloride. The sequence and the proteolytic cleavage pattern suggest a three domain structure. The N-terminal 300 residues (pI 8.7) harbor a clathrin binding site. An acidic middle domain (pI 3.6, 450 residues), interrupted by an uncharged alanine rich segment of 59 residues, seems responsible for the anomalous physical properties of AP180. The C-terminal domain (166 residues) has a pI of 10.4. AP180 mRNA is restricted to neuronal sources. AP180 shows no significant homology to known clathrin binding proteins but is nearly identical to a mouse phosphoprotein (F1-20). This protein, localized to synaptic termini, has so far been of unknown function.

H 315 EXPRESSION AND SECRETION OF HUMAN FIBRINOGEN IN YEAST

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Human fibrinogen is a structural protein of the plasma which is involved in blood coagulation. It has an $\alpha_2\beta_2\gamma_2$ subunit composition, a molecular weight of 340,000 and contains 29 disulfide bonds. Assembly and secretion of fibrinogen are poorly understood. It appears that synthesis of the β chain is limiting for assembly in human liver cells. Each fibrinogen polypeptide chain contains an N-terminal signal sequence for entry into the ER where it is assembled. Fibrinogen is secreted from the cell and can be converted to a high order fibrin structure by the action of thrombin and factor XIIIa. Thrombin cleaves short peptide sequences from the N-termini of the α and β chains which results in interactions between different fibrinogen molecules. Factor XIIIa is a transglutaminase that crosslinks fibrin monomers together to strengthen the clot. We have expressed and secreted each of the individual fibrinogen chains using either the MFα1 leader or the native fibrinogen signal sequences. However, secretion is not efficient as most of the protein expressed accumulates intracellularly. A yeast strain expressing all three fibrinogen chains has been constructed. We are currently testing whether yeast can assemble and secrete this complex molecule.

H 316 INTRACELLULAR TRAFFICKING OF HUMAN INSULIN-RELATED PROTEINS IN YEAST
Chris Raymond and Don Foster, Department of Molecular and Cellular Biology, ZymoGenetics, Inc., 4225 Roosevelt Way NE, Seattle, WA 98115
One of the major research goals at ZymoGenetics, Inc. is to produce therapeutically significant human proteins in *Saccharomyces cerevisiae.* We are currently investigating the biosynthesis and secretion of human insulin-related proteins (HIPs) in this heterologous expression system. In the steady state, roughly half of the HIP produced by the yeast host strain is retained intracellularly. Analysis of intracellular HIP by SDS-PAGE and Western blotting suggests that much of the protein resides in either the Kex2p compartment or a compartment distal to this late-Golgi organelle. Localization of HIP by indirect immunofluorescence reveals that a substantial pool of HIP, if not the majority of the HIP, is present in the vacuole. We are currently attempting to understand how HIP is directed to the vacuolar compartment. One model is that HIP carries a vacuolar targeting signal. By this model, some HIP is secreted because it escapes an HIP-saturated vacuolar sorting apparatus and is secreted by default. A second model is that high levels of HIP expression result in delivery to the vacuole by an uncharacterized pathway. These models can be differentiated by examining the localization of HIP at lower levels of HIP expression. An increase in the percentage of vacuole-localized HIP would favor the first model, while an increase in the percentage of secreted HIP at lower levels of expression would favor the second. Data on the localization of HIP at lower expression levels will be presented.

H 317 STRATEGIES FOR EFFICIENT SECRETION OF HETEROLOGOUS PROTEINS BY THE FILAMENTOUS FUNGUS *ASPERGILLUS NIGER*, Cees A.M.J.J. van den Hondel, Ineke E. Mattern, Jim R. Kinghorn, Roland Contreras and Cees A.M.J.J. van den Hondel and Martien P. Broekhuijsen, Medical Biological Laboratory TNO, P.O.box 45, 2280 AA Rijswijk, The Netherlands.

Several filamentous fungal species, like *Aspergillus niger* are capable to produce large amounts of extracellular enzymes. However, production of heterologous proteins in *A. niger* is still rather inefficient. Possible problems arise from proteolytic degradation by (extracellular) enzymes from the host-organism and/or inefficient processing through the secretion pathway.

To develop improved methods we studied the production of human interleukin-6 (IL6) in *A. niger*. *In vitro* experiments with culture medium revealed that IL6 was rapidly degraded. Therefore we isolated several protease-deficient mutants of *A. niger*, which resulted in considerable less degradation of IL6 *in vitro*. One of these mutants was transformed with different IL6 expression vectors. First, IL6 was expressed using different signal sequences. The resulting transformants did not produce detectable amounts of IL6, despite high levels of mRNA. We hypothesized that IL6 was not efficiently processed through the secretion pathway. Therefore, IL6 was expressed as a fusion protein with glucoamylase, being a protein which is very well secreted by *A. niger* and expression of which can easily be measured. To obtain mature IL6, a KEX2 cleavage site was inserted between the glucoamylase and IL6 sequences. In this way, mature, active IL6 was obtained from *A. niger* transformants up to 15 mg/l, an increase of at least 1000* compared to the original constructs.

H 318 SELECTIVE RETENTION IN THE YEAST ER OF SECRETORY PROTEINS BY IN VIVO REDUCTION **Marja Makarow, Eija Jämsä and Marjo Simonen Inst. Biotechn., Univ. Helsinki, Helsinki, Finland**
We are studying the consequences on secretion of interfering with the folding of proteins in the yeast ER. As a marker we use the hsp150 protein, whose gene we have cloned and characterized. Hsp-150 is secreted fast and efficiently to the growth medium. When it is synthesized in the presence of the reducing agent dithiothreitol (DTT), it is retained specifically in the ER and/or vesicles budding from the ER membrane. When native hsp150 is arrested in the Golgi or secretory vesicles, and thereafter reduced, secretion is not effected. Secretion of invertase is not inhibited by DTT. After removal of DTT, hsp150 is chased to the medium with a half time of 10 min, which is 5-times slower than that of normal secretion. The hsp150 protein is largely composed of a highly repetitive region, which occurs as a random coil. This portion is followed by a C-terminal part which has all 4 cysteines of hsp150. They apparently form disulphide bridges, since a native form of hsp150 could be differentiated in SDS-PAGE from a reduced form obtained after in vivo DTT-treatment. Hsp150 is oligomerized in the ER, but this is not affected by DTT. A conformational change resulting from DTT-treatment was revealed by using a fusion protein where the Cys-containing C-terminal part of hsp150 was replaced by β-lactamase which contains two cysteins. The fusion protein is efficiently secreted and enzymatically active. When synthesized in the presence of DTT, it remained intracellular and was inactive. Upon chase after removal of DTT, it gained secretion-competence, but remained inactive. Currently we are studying wether BiP or other proteins are associated to reduced hsp150, and hope to develope a screening assay using the fusion protein to isolate mutants defective in retention of malfolded proteins.

H 319 MOLECULAR ANALYSIS OF STE6, THE YEAST a-FACTOR TRANSPORTER, Carol Berkower and Susan Michaelis, Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, MD 21205
The STE6 protein of *Saccharomyces cerevisiae* mediates export of the a-factor mating pheromone via a novel pathway. STE6 is composed of two homologous halves, each containing six membrane spanning segments and one putative ATP binding domain. STE6 belongs to a superfamily of transport proteins designated the ATP binding cassette (ABC) proteins, which share significant overall structure. ABC family members include MDR, the mammalian multidrug resistance protein, and CFTR, the protein defective in patients with cystic fibrosis. The STE6, CFTR and MDR proteins are each encoded by a single large polypeptide. In contrast, bacterial family members encode individual domains of a single transporter on separate genes, reflecting the modular organization of these pumps.

To examine structure-function relationships in STE6, we severed the coding region of the *STE6* gene between its two homologous halves, and showed that the resulting half-molecules could regenerate a functional transporter (Berkower and Michaelis. 1991. *EMBO J.* 10:3777). More recently, we have generated one-quarter and three-quarter molecules. The N-terminal one-quarter molecule, containing six predicted membrane spans, can regenerate a functional transporter in combination with the remaining three-quarter molecule (which lacks transport activity on its own). This suggests that the N-terminal hydrophobic domain of STE6 can provide function, even when it is not covalently linked to the rest of the transporter. However, the C-terminal quarter-molecule of STE6 cannot reassemble with the N-terminal three-quarter molecule to reconstitute a functional transporter, which is in contrast to several bacterial permeases, whose four domains are all encoded independently. We are examining the ability of the isolated half- and quarter-molecules to bind the radiolabelled ATP analogue, [α-^{32}P]8-azido-ATP. We have observed [α-^{32}P]8-azido-ATP binding to full-length STE6. We are also performing binding assays on several STE6 point mutants which appear to be defective in their ability to utilize ATP.

STE6 has a short half-life, of around 30 minutes. To determine the particular region(s) of STE6 which target it for degradation, as well as to identify trans-acting components of the degradation pathway, we are examining instability of STE6 half- and quarter-molecules. The C-terminal quarter-molecule does not localize to the membrane and, interestingly, is much more stable than full-length STE6. The effects of mutations in vacuolar proteases on the half-life of STE6 will also be discussed.

H 320 EFFECT OF BREFELDIN A ON PROTEIN TRANSPORT IN *Saccharomyces cerevisiae*. Todd R. Graham*, Peggy A. Scott, and Scott D. Emr, Division of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, CA 92093-0668 and *Department of Molecular Biology, Vanderbilt University, Nashville TN, 37235

We have found that brefeldin A (BFA) inhibited the growth of the drug-permeable *ise1* mutant of *Saccharomyces cerevisiae*. Genetic mapping indicated that *ise1* was allelic to *erg6*, a gene required for the biosynthesis of the principal membrane sterol of yeast, ergosterol. Treatment of labeled *ise1* cells with BFA resulted in an immediate block in protein transport through the secretory pathway. Carboxypeptidase Y (CPY) and α-factor (αf) accumulated as both the core glycoslated (ER) and α1-6 mannosylated (early Golgi) forms in drug treated cells. We found that transport of CPY from medial and late Golgi compartments to the vacuole was unaffected by conditions of BFA treatment that completely inhibited protein transport from the ER and early Golgi. After 1 hour in BFA, 70-80% of the cells were still viable, but rapidly lost viability thereafter. The effects of BFA on the secetory pathway were also reversible after brief exposure (<40 min.) to the drug. We suggest that the primary effect of BFA in *Saccharomyces cerevisiae* is restricted to the ER and the α1-6 mannosyltransferase compartment of the Golgi complex.

H 321 THE ESSENTIAL FUNCTIONS OF ACTIN AND END3 IN THE INTERNALIZATION STEP OF ENDOCYTOSIS IN YEAST.

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Alpha-factor pheromone is internalized by receptor-mediated endocytosis in *Saccharomyces cerevisiae* a cells.Three distinct steps can be distinguished and measured: binding of alpha-factor to its receptor, pheromone internalisation, and subsequent transport to the vacuole where the pheromone is degraded (Singer and Riezman 1990). This type of analysis has allowed the classification of endocytic mutants into two groups: those affected in the uptake step, and those affected later in the pathway, thereby abolishing the transfer of alpha-factor to the vacuole.

Actin has an essential function in the uptake step because the temperature sensitive mutant, *act1-1*, is completely defective for alpha-factor internalization at 37°C. Controls performed with *sec* mutants show that the *act1-1* endocytosis defect is not due to the effect of the mutation on the secretory pathway. The later steps of the endocytic pathway are not grossly affected in the *act1-1* mutant because alpha-factor internalized at the permissive temperature and trapped in the endosomes, can be subsequently delivered to the vacuole at 37°C. The actin mutants are also defective in vacuolar lucifer yellow accumulation, indicating a defect in fluid phase endocytosis.

Other mutants affected in this uptake step have been isolated (Raths et al, J.C.B, in press). One of these strains, *end3*, is thermosensitive for growth and defective for alpha-factor uptake and vacuolar lucifer yellow accumulation at 24°C and 37°C. Disruption of the gene results in the same phenotype. End3p (349 aa) possesses two regions that are important for its function, the N-terminal domain (residues 1 to 103) and a short stretch of residues in the C-terminal domain (residues 309 to 327). In contrast, the two central heptad-repeat regions are not required for function *in vivo*. The *end3* mutants present a completely different pattern of actin staining than wild-type cells, suggesting that End3p plays a role in actin localization for endocytosis.

H 322 EARLY *sec* MUTANTS DO NOT BLOCK RECEPTOR-MEDIATED INTERNALIZATION OF α-FACTOR BUT ARE DEFECTIVE IN TRANSPORT OF α-FACTOR TO THE VACUOLE.

Linda Hicke and Howard Riezman, Department of Biochemistry, Biocenter, University of Basel, CH-4056 Basel, Switzerland

We would like to identify proteins that function in the endocytic pathway in yeast. One approach is to determine whether proteins required for membrane transport in the secretory pathway, encoded by *SEC* genes, also carry out similar functions for membrane transport in the endocytic pathway. We have decided to analyze the ER to Golgi *sec* mutants, as they block the secretory pathway at the first step requiring membrane transport and are therefore good candidates for proteins that function in other membrane transport processes. Two distinct steps in the yeast endocytic pathway can be assayed, internalization of α-factor into the cell by receptor-mediated endocytosis, and transport of internalized α-factor through endocytic intermediates to the vacuole. We have found that none of the early *sec* mutants that have been tested (*sec7*, *sec12*, *sec13*, *sec14*, *sec16*, *sec17*, *sec18*, *sec19*, *sec20*, *sec21*, *sec22*, *sec23* and *ypt1*) block internalization of α-factor after a 20 min preincubation at the nonpermissive temperature (37°C). In contrast, in preliminary experiments to determine the efficiency with which internalized α-factor is transported to the vacuole and degraded in the mutants, *sec12*, *sec16*, *sec17*, *sec18*, *sec20*, *sec23* and *ypt1* were 2.5-4 fold less efficient than wildtype cells in degrading α-factor at 37°C. *sec21* showed no defect in α-factor degradation. *sec18* was further analyzed for its ability to transport internalized, radiolabeled α-factor through two kinetically-related endocytic intermediates (Singer *et al.*, submitted). In wildtype cells incubated at 37°C, α-factor traversed through these intermediates and was no longer detected in them after 60 min. In *sec18* incubated at 37°C, however, α-factor accumulated in and did not chase from an early endosomal compartment even after 90 min.

None of the *SEC* gene products involved in the budding or fusion of transport vesicles from the ER are required for α-factor internalization, indicating that the budding of endocytic vesicles from the plasma membrane may be a fundamentally different process than budding that occurs during the secretory pathway. In addition, we conclude that Sec18p, the yeast NSF homolog, is required for at least one fusion event that occurs along the endocytic pathway. It is surprising that so many additional *sec* mutants also appear to block α-factor degradation. We are in the process of determining whether these Sec proteins are also directly required for endocytic transport.

H 323 NOVEL YEAST MUTANTS DEFECTIVE IN DIFFERENT STAGES OF ENDOCYTIC MEMBRANE TRAFFIC,

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Mutants of *Saccharomyces cerevisiae* which are defective in vesicular transport from the cell surface to the lysosome-like vacuole are inviable if they carry a disruption of the gene encoding the catalytic subunit of the vacuolar ATPase (*VAT2*). In yeast cells deficient in vacuolar ATPase, fluid phase endocytosis may provide an alternative pathway by which the lumen of the vacuole and other endocytic compartments can be acidified. We have used this synthetic lethality with *vat2* mutants to devise a genetic screen for novel endocytosis (*end*) mutants. We isolated from a pool of mutagenised cells 18 mutants which are inviable when they carry a disrupted copy of *VAT2* (*vat2del*). Of these, 7 are defective for accumulation of the fluid phase endocytic marker lucifer yellow CH in the vacuole and therefore have an End$^-$ phenotype. Thus far we have analysed three *end* mutants for endocytosis of α-factor. One is defective for internalisation at the plasma membrane and two internalise normally, but appear to be blocked or delayed in delivery of the internalised α-factor to the vacuole. All of the *end* mutants possess a vacuole of normal morphology and most are temperature-sensitive for growth. Analysis of these mutants will provide insights into the molecular mechanisms underlying endocytic membrane transport.

H 324 CHARACTERIZATION OF PROTEINS ASSOCIATED WITH Sec7p IN EXTRACTS OF *SACCHAROMYCES CEREVISIAE*,

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Sec7p, encoded by the yeast SEC7 gene, is a 230 kDa acidic phosphoprotein that is required for vesicular transport from the ER and between the different compartments of the yeast Golgi apparatus (Nature 355: 173, 1992). Sec7p is found on the surface of transport vesicles, intracellular organelles, and as part of a soluble cytosolic complex. The cycling of Sec7p between its soluble and membrane-associated states may depend on the action of the Sec7p-associated components in the Sec7p complexes.

Non-denaturing immuno-precipitation of yeast cytosol followed by polyacrylamide gel electrophoresis and Coomassie blue staining show that proteins of 230 (Sec7p), 83, 60, and 32 kDa are specifically precipitated with antibody to Sec7p (Sec7-Ab). A 25 kDa GTP-binding protein is also associated with Sec7p, as demonstrated by GTP-ligand binding blots. Additional proteins are associated with Sec7p, as shown by experiments in which lysates of radiolabeled spheroplasts are covalently attached with a cleavable bifunctional crosslinking reagent and immunoprecipitated with Sec7-Ab. In particular, a protein of 38 kDa is associated with Sec7p in stoichiometric amounts, whereas another protein of ~100 kDa is found crosslinked to Sec7p in sub-stoichiometric amounts. Hence, some proteins associated with Sec7p may regulate vesicular traffic at unique, rather than multiple, stages of the yeast secretory pathway.

H 325 PURIFICATION AND CHARACTERIZATION OF SARlp, A SMALL GTPase REQUIRED FOR TRANSPORT VESICLE FORMATION FROM THE ER

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The *SEC12*, *SAR1* and *SEC23* genes are required for transport vesicle formation from the ER in yeast. Secl2p is an integral ER membrane glycoprotein with a prominent N-terminal cytosolic domain. *SAR1* was discovered as a multi-copy suppressor of a *sec12^ts* strain and found to encode a 21 kD GTPase. An *in vitro* assay that measures the formation of a vesicle intermediate in ER to Golgi transport has been devised that is dependent on the addition of Sarlp. This assay utilizes membranes prepared from wild-type cells and a cytosol depleted of Sarlp by gel-filtration chromatography. This Sarlp-depleted fraction requires the addition of Sarlp and GTP to support vesicle budding. Preloading Sarlp with GTPγS inhibits Sarlp function in the vesicle formation assay. We have employed this assay to purify functional Sarlp from a yeast cytosol after overexpression of *SAR1* under a galactose regulated promoter. Characterization of the purified protein demonstrates Sarlp binds guanine nucleotide and contains GTPase activity. Detectable nucleotide exchange and hydrolysis rates are increased in the presence of Mg^{2+} and non-ionic detergents or phospholipids. Interestingly, purified Sec23p stimulates GTP hydrolysis by Sarlp at least ten fold. Further, the cytosolic domain of Secl2p increases the nucleotide exchange rate of Sarlp. These results suggest that a cycle of GTP hydrolysis and nucleotide exchange by Sarlp is required for transport vesicle budding from the ER and this cycle is regulated by the Secl2p and Sec23p proteins.

H 326 *SEC21*, AN ESSENTIAL GENE REQUIRED FOR ER TO GOLGI TRANSPORT, ENCODES A SUBUNIT OF A YEAST COATOMER,

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SEC21 is an essential gene required for the transport of secretory, vacuolar and plasma membrane proteins from the endoplasmic reticulum to the Golgi apparatus in the yeast *Saccharomyces cerevisiae*. A temperature sensitive mutant, *sec21-1*, accumulates unprocessed, core glycosylated precursors of secretory and vacuolar proteins when shifted to the non-permissive temperature. The *SEC21* gene encodes a 105 kDal cytoplasmic protein, and fractionation experiments have shown that some of the Sec21 protein is peripherally associated with the cytoplasmic face of a sedimentable membrane fraction. Gel filtration chromatography of cytosol followed by immunoblot analysis indicates that cytosolic Sec21 protein has a native relative molecular mass of 700 - 800 kDal, suggesting that the Sec21 protein exists in a large protein complex either with itself or other proteins. Purification of this protein complex to homogeneity has shown that the Sec21 protein is indeed associated with 5 other polypeptides. This Sec21 protein-containing complex has a similar subunit composition, size, and purification properties as mammalian coatomer, a complex that has been postulated to be the coat protomer of intra-Golgi transport vesicles. An antibody against the 110 kDal mammalian coatomer subunit, βCOP, recognizes the corresponding 110 kDal subunit of the yeast complex. This is the first demonstration of a requirement for a coatomer complex in intercompartmental transport *in vivo*. The precise role of the yeast coatomer in ER to Golgi transport is currently being explored using *in vitro*.

H 327 THE SEC13p COMPLEX AND RECONSTITUTION OF VESICLE BUDDING FROM THE ER WITH PURIFIED PROTEINS,

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The vesicle mediated movement of proteins through the secretory pathway from the endoplasmic reticulum (ER) to the Golgi apparatus in *Saccharomyces cerevisiae* requires the *SEC13* gene product. *SEC13* encodes a 34,000 MW protein that exists both peripherally associated with the membrane fraction and free in the cytosol. Further fractionation by gel filtration chromatography shows that cytosolic Sec13p exists in a monomeric form and in a high molecular weight complex of approximately 800,000 MW. A SEC13-mouse dihydrofolate reductase hybrid protein (Sec13p-dhfr) was constructed that functions *in vivo* in both a temperature sensitive and a null *sec13* mutant background. Like Sec13p, cytosolic Sec13p-dhfr exists as a monomer and in a high molecular weight form. Methotrexate-agarose affinity chromatography has facilitated the purification of both the monomer and the high molecular weight form of the hybrid protein. The high molecular weight form of Sec13p-dhfr consists of two subunits: Sec13p-dhfr and a 150,000 MW protein (p150). Native immunoprecipitations from radiolabeled wild type cells reveal that Sec13p specifically associates with a 150,000 MW protein. Anti-p150 Fab fragments and anti-Sec13p Fab fragments inhibit vesicle budding in a semi-intact cell assay. Using the semi-intact cell transport vesicle budding assay we find that the Sec13p-dhfr/p150 complex, but not Sec13p-dhfr monomer, can replace the cytosolic requirement of this reaction in combination with two previously characterized protein fractions. Thus vesicle budding from the ER requires three cytosolic protein fractions: Sarlp, a small GTP binding protein, the Sec23p/Sec24p protein complex and the Sec13p/p150 protein complex.

H 328 NEW YEAST SECRETORY MUTANTS DEFECTIVE IN ER TO GOLGI TRANSPORT DISPLAY A UNIQUE MEMBRANE ACCUMULATION MORPHOLOGY,

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We have isolated thirteen new temperature-sensitive yeast mutants defective in ER to Golgi transport using a colony blotting screen that detects uncleaved α-factor precursor (Wuestehube *et. al.*, 1991, J. Cell Biol. 115:475a). The new mutants define at least five new complementation groups. In addition we have isolated new alleles of previously identified ER-Golgi genes *SEC13, SEC16, SEC23* and *USO1*. Biochemical studies show that the mutants accumulate several soluble secretory proteins *in vivo* including α-factor precursor, invertase and the ER-modified p1 form of carboxypeptidase Y. The newly identified genes may function in concert with other *SEC* genes since double mutant analysis shows that the new mutants display specific synthetic lethal interactions with previously isolated ER-Golgi secretory mutants. Thin section electron microscopy analysis reveals that whereas some of the mutants show the classic morphology of accumulated 50 nm vesicles and continuous ER membrane, others also accumulate fragments of membrane in the cytosol. These membrane fragments may be ER-derived indicating a requirement of these gene products for maintaining the integrity of the ER membrane. Alternatively the morphology of these mutants may reveal a post-ER, pre-Golgi membrane compartment. The nature of these membrane fragments currently is under investigation.

H 329 IDENTIFICATION AND CHARACTERIZATION OF PROTEINS INTERACTING WITH THE SECRETORY PROTEIN PREPROALPHAFACTOR IN THE LUMEN OF THE ENDOPLASMIC RETICULUM OF *S. CEREVISIAE*.

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We asked which proteins interact with secretory proteins in the lumen of the endoplasmic reticulum (ER) and in ER to Golgi transport vesicles in yeast using a chemical crosslinking approach. Radio-labelled preproalphafactor was translocated into the ER of semi-intact yeast cells *in vitro*. The cells were incubated in the presence of cytosol and ATP to allow for budding of transport vesicles and cells were separated from the vesicles by differential centrifugation. Both the vesicle fraction and the semi-intact cells containing the ER were treated with the amino-reactive, homobifuntional, membrane-permeable crosslinker disuccinimidyl suberate and proteins crosslinked to proalphafactor were either precipitated with anti-alphafactor antibody or with Concanavalin A-Sepharose. Two major crosslinking products of 45 kDa and 50 kDa molecular weight were identified in the semi-intact cells, but not in the vesicle fraction. Both crosslinking products were resistant to trypsin digestion and extractable from the cells with carbonate which suggests they reside in the lumen of the ER. We are now characterizing these proteins employing reversible crosslinkers, immunoprecipitation with antibodies against known ER proteins and mutants defective in export from the ER in order to elucidate their function.

H 330 Sar1 PROTEIN: A NEW CLASS OF SMALL GTPase THAT FUNCTIONS IN VESICLE FORMATION.

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SAR1, a yeast gene first identified as a multicopy suppressor of the *sec12* ts mutation, encodes a unique type of 21 kDa GTP-binding protein that shows about 20-25% identity to Ras and Ypt (Rab) family and 30-35% identity to ARF family proteins[1]. We have demonstrated that Sar1p is mostly localized on the cytoplasmic surface of the ER membrane[2] and cooperates with Sec12p (ER membrane protein) in the ER-to-Golgi protein transport[1,2]. Interestingly, Sar1p has no sites for any known type of lipidic modifications at either N- or C-terminus unlike other members of the Ras-superfamily, but is very tightly associated with the membrane[2]. Taking the advantage that the purified Sar1p can suppress the temperature sensitive transport of the *sec12* membranes *in vitro*, we have analyzed the function of Sar1p from the viewpoint of the role of GTP[3]. Our conclusion led from this cell-free assay is that Sar1p is involved in the formation of transport vesicles from the ER, and that the hydrolysis of GTP is required after the release of the vesicles (see also the abstract of Oka and Nakano). Like many other small GTPases, other components such as the GTPase-activating protein and the GDP/GTP exchanger may regulate the Sar1p function. Genetic studies have hinted that Sec12p, Sec13p, Sec16p and Sec23p are candidates for these components and in fact two of them are shown to have such activities (Yoshihisa, Barlowe and Schekman, personal communication). We have introduced random and site-directed point mutations in *SAR1* and tested if they could affect the Sar1p function. So far we have obtained three ts alleles and four dominant lethals, which will be useful tools in understanding the execution points of Sar1p function both by *in vivo* and *in vitro* approaches.

1. Nakano and Muramatsu (1989) *J. Cell Biol.* **109**:2677-2691
2. Nishikawa and Nakano (1991) *Biochim. Biophys. Acta* **1093**:135-143
3. Oka, Nishikawa and Nakano (1991) *J. Cell Biol.* **114**:671-679

H 331 Sar1p-GTPγS ACCUMULATES VESICLES THAT ARE FUNCTIONAL INTERMEDIATE OF THE ER-TO-GOLGI TRANSPORT

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In the yeast secretory pathway, a small GTP-binding protein Sar1p and an ER membrane protein Sec12p cooperate in ER-to-Golgi transport. Using a transport assay based on the semi-intact cell system, we reproduced the temperature sensitivity of the *sec12* membranes and its suppression by Sar1p. To investigate the functional roles of these proteins, we set up an assay to quantify the formation of transport vesicles by measuring the amount of trypsin-resistant pro-α-factor released from the *sec12* membranes. At the restrictive temperature, the *sec12* membranes failed to release vesicles. This defect was completely suppressed by the addition of Sar1p-GTP. Sar1p-GTPγS also suppressed the defect of vesicle formation but inhibited further transport to the Golgi. This indicates that GTP hydrolysis by Sar1p is not required for suppression of the *sec12* defect but is essential for the transport of the released vesicles to the Golgi apparatus. Sedimentation analysis on a sucrose density gradient showed that vesicles generated by Sar1p-GTPγS migrated as a single peak at the density of 29% (w/v) sucrose. When the peak fraction was further subjected to flotation analysis, the vesicles again formed a single peak at the density of 37% (w/v) sucrose. As a demonstration that the vesicles accumulated by Sar1p-GTPγS are the functional intermediate of ER-to-Golgi transport, we have developed a chase assay in which the vesicle contents are delivered to the Golgi by reincubation of the separated vesicles with semi-intact cells. This reaction was dependent on ATP, cytosol and acceptor membranes. The *sec12* semi-intact cells were capable of accepting the vesicles even at the restrictive temperature. This suggests that the *sec12* membranes have a defect only in the release of the intermediate vesicles from the ER.

H 332 ISOLATION OF YEAST MUTANTS DEFECTIVE IN THE ER RETENTION OF Sec12 PROTEIN

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The yeast *SEC12* gene product (Sec12p) is an integral membrane protein required for the formation of transport vesicles from the endoplasmic reticulum (ER). Although this protein is exclusively localized in the ER, a significant fraction is modified by an early Golgi enzyme, α-1,6 mannosyltransferase. These observations suggest that Sec12p is retained in the ER by membrane recycling between the ER and the Golgi. Sec12p does not acquire α-1,3 mannosyl linkages which are believed to be added in the later compartment of the Golgi, suggesting that this recycling occurs between the ER and the early Golgi.

In order to investigate the ER retention and recycling mechanism of Sec12p, we have taken a genetic approach. In a mutant defective in the ER retention of Sec12p, Sec12-Mfα1 fusion protein as well as Sec12p are expected to transported to the late Golgi compartment by bulk flow. Then, by the action of Kex2 protease, a late Golgi protein, mature α-factor will be produced from the fusion protein. By screening mutagenized yeast cells, we isolated four recessive mutant clones that showed fusion-protein-dependent secretion of α-factor. They fell into two complementation groups and were designated *rer1* and *rer2*. Pulse-chase analysis showed that, in the *rer1* mutant, Sec12p received carbohydrate modifications of late Golgi compartment. In this mutant, we also observed mislocalization of Sec12-inveratase fusion protein to the cell surface. These results suggest that the *rer1* mutant is defective in the ER retention of Sec12p. In contrast, this mutant is normal in the retention of a soluble ER protein, BiP, suggesting that soluble and membrane proteins are retained in the ER by distinct mechanisms.

H 333 THE *och2* MUTATION IS A ts ALLELE OF *SAR1*, WHICH IS A SECRETION-ASSOCIATED AND *ras*-SUPERFAMILY-RELATED GENE, AND CAUSES BYPASS OF GOLGI APPARATUS IN PROTEIN SECRETION. Yoh-ichi Shimma, Makoto Enatsu, and Yoshifumi Jigami. Division of Biological Chemistry, National Chemical Laboratory for Industry, M.I.T.I. Tsukuba, Ibaraki 305, Japan.

The *och* mutants, which are deficient in outer-mannose-chain elongation in N-linked glycosylation and show a temperature-sensitive growth, have been isolated through tritium-mannose-suicide method (Nagasu *et al.*, YEAST 1992). The *och1* mutation confers a core-glycosylated form whose size is slightly larger than that of ER form, and *OCH1* gene was found to encode mannosyltransferase (Nakayama *et al.*, EMBO J. 1992). The *och2* mutant cells produced ER form glycoproteins and secreted their glycoproteins to the periplasmic space. To examine a secretory pathway in the *och2* cells, we have constructed a series of double mutants between *och2* and several *sec* mutations. In contrast to that the single *sec* mutant, *sec22, sec7*, and *sec14*, which is deficient in the protein transport in the Golgi apparatus, did not secrete invertase to the cell surface, three double mutants, *och2 sec22, och2 sec7*, and *och2 sec14*, secreted invertase to the cell surface like the *och2* single mutant cells. This indicates that in *och2* cells proteins are secreted by bypassing the Golgi apparatus, where mannose-outer-chain addition occurs. *OCH2* gene was cloned by complementation of *och2* ts phenotype. The DNA sequencing analysis revealed that *OCH2* gene is identical with *SAR1* gene, which encodes a ras-homologous GTP-binding protein. The *SAR1* protein is involved in pinching off of transport vesicles from the ER, and secretory proteins are accumulated in the ER (Nakano *et al.* 1990). Thus, the *och2* cells showed a different phenotype from *sar1* mutant in protein secretion. The analysis of the mutation point of *och2* gene revealed that the *och2* mutant gene has a single base change from C to T at the third position of TACTAAC box, which is a common branching sequence of yeast intron necessary for RNA splicing. We are now addressing the relation between the *och2* mutation at the TACTAAC box and the bypass of the Golgi apparatus in protein secretion.

H 334 ISOLATION OF AN *ARABIDOPSIS THALIANA* GENE HOMOLOG TO A YEAST GENE INVOLVED IN VACUOLAR PROTEIN SORTING. Peter Welters, Scott D. Emr and Maarten J. Chrispeels, Department of Biology, University of California San Diego, La Jolla, CA 92093-0116

Like in yeast, vacuolar targeting information in plant proteins is an intrinsic property of the polypeptide chain. N-terminal and C-terminal pro-domains of vacuolar plant proteins have been shown to be both necessary and sufficient for vacuolar targeting; however, nothing is known about the underlying mechanism of this process in plants. To acquire more information about vacuolar sorting in plant cells we attempted to isolate the plant homolog of yeast *VPS*34, a vacuolar sorting gene that encodes a phosphatidylinositol 3-kinase (PI3-kinase). Using PCR with oligonucleotides to conserved regions of PI-kinases of yeast and mammalian origin and hybridization with the appropriate domain of the yeast *VPS*34 gene we found an *A. thaliana* sequence with 49% identity to the yeast Vps34 protein domain but only 27% identity to the mammalian PI3-kinase between the conserved primer region. This PCR-derived clone was used to screen a cDNA library. Positive clones will be used to complement yeast vps34 mutants. Overexpression and antisense studies in *A. thaliana* are planned.

PI3-kinase activity is associated with the yeast *VPS*34 gene product. However, this enzymatic activity has never been detected in plants (Xu et al., Plant Cell, 1992). Our finding of the plant homolog of *VPS*34 is therefore of special interest, not only to understand the vacuolar targeting mechanism but also to demonstrate conservation of transport mechanisms in yeast, animals and plants.

H 335 CHARACTERIZATION OF THE PHOSPHATIDYL-INOSITOL 3-KINASE REQUIRED FOR VACUOLAR PROTEIN SORTING IN YEAST. Kaoru Takegawa, Peter V. Schu, Jeffrey H. Stack and Scott D. Emr, Division of Cellular and Molecular Medicine, Howard Hughes Medical Institute, UCSD School of Medicine, La Jolla, CA 92093-0668

In the yeast *Saccharomyces cerevisiae*, the application of several genetic selections has resulted in the identification of more than 40 *vps* (vacuolar protein sorting) complementation groups. The *vps* mutants exhibit defects in vacuolar protein localization and processing. The *VPS*34 gene encodes an 875 amino acid protein which shares significant sequence similarity with the recently identified catalytic subunit of bovine phosphatidylinositol 3-kinase (PI3-kinase). PI3-kinase activity can be detected in a wild-type yeast cell extracts, however, extracts from a strain deleted for the *VPS*34 gene lack detectable PI3-kinase activity. Moreover, overexpression of Vps34p results in an increase in PI3-kinase activity, and this activity can be specifically precipitated with Vps34p-antisera. In addition, point mutations altering highly conserved residues within the Vps34 kinase domain result in inactivation of Vps34p/PI3-kinase and the missorting which causes secretion of vacuolar proteins. These data indicate that *VPS*34 encodes PI3-kinase, and the formation of 3-phosphorylated PI-phosphate(s) is essential for vacuolar protein sorting in yeast.

PI3-kinase activity has also been detected in extracts from the fission yeast *Schizosaccharomyces pombe*. Three oligodeoxyribonucleotide primers corresponding to three conserved amino acid sequences between Vps34p and bovine PI3-kinase were used in a polymerase chain reaction (PCR) to amplify *S. pombe* DNA. The PCR product has been used as a hybridization probe to clone a putative PI3-kinase gene from a *S. pombe* genomic library. The sequence of the *S. pombe* PI3-kinase is very close to that of Vps34p (50% amino acid identity), compared with 30% amino acid identity to the bovine PI3-kinase. This similarity is particularly striking in a 60 amino acid domain where the identity between *S. pombe* and Vps34p is more than 70%. This domain contains a subset of sequence motifs that are highly conserved in all protein kinases.

H 336 CHARACTERIZATION OF THE VAC2 GENE AND ITS ROLE IN VACUOLE INHERITANCE DURING YEAST BUDDING.

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During mitotic division in *Saccharomyces cerevisae*, the newly formed bud inherits a vacuole directly from the mother cell. Cytological studies demonstrate that the formation of bud vacuoles by this pathway is both spatially regulated and tightly coordinated with the cell cycle. We previously reported the isolation of a vacuole inheritance (vac) mutant, *vac2*, that interfers with the transfer of maternal vacuoles into emerging buds. At 37°C, a mutant strain containing a temperature-sensitive allele of VAC2 (*vac2-1*) produces large buds and daughter cells that lack wild-type vacuoles. Although the *vac2* mutation specifically disrupts the vacuole inheritance pathway in yeast, it does not interfere with the partitioning of nuclei or mitochondria into daughter cells.

We have cloned the wild-type VAC2 gene by complementation of the *vac2-1* mutation. Studies to date indicate that the VAC2 gene resides on chromosome XI and encodes a novel protein. Expression of the C-terminal 277 amino acids of the VAC2p on a centromere-based plasmid (YCp50) partially rescues the vacuole inheritance defect in the *vac2-1* mutant strain. Studies designed to investigate the precise role of the VAC2p in vacuole inheritance are currently in progress.

H 337 THE ROLE OF COMPARTMENT ACIDIFICATION IN SORTING OF SOLUBLE AND MEMBRANE PROTEINS TO THE YEAST VACUOLE

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The vacuole of the yeast *Saccharomyces cerevisiae* contains a proton-translocating vacuolar-type ATPase (V-ATPase) which acidifies the vacuolar lumen. The yeast V-ATPase is a member of a larger class of V-ATPases with homologs in plant and animal systems. This family of ATPases is characterized by a peripherally associated subcomplex (V_1) which includes subunits with ATP-binding and catalytic domains (VATA, 69 kD; VATB, 57 kD; VATC, 42 kD; VMA4, 31 kD). In addition, there are at least two membrane proteins which are components of the proton-translocating pore (V_O; VAT<u>c</u>, 16 kD; VPH1, 100 kD) . The role of compartment acidification in the sorting of vacuolar proteins has been investigated using both biochemical and molecular genetic methods. Kinetic analyses using an inhibitor of the V-ATPase or chemicals that cause an increase in the vacuolar pH result in missorting of the soluble hydrolases carboxypeptidase Y (CPY) and proteinase A (PrA) under these conditions. Disruptions in genes encoding the A, B or <u>c</u> subunits of the V-ATPase also result in accumulation of precursor forms of soluble vacuolar proteins. In contrast, the vacuolar membrane protein alkaline phosphatase (ALP) is relatively insensitive to acidification defects; precursor accumulation only occurs under chronic conditions such as those which exist in *vat* mutant strains. These results suggest that vacuolar targeting of ALP is not directly affected by compartment acidification. We have extended the analysis by performing kinetic and steady-state experiments using strains with disruptions in the *VATC* and *VMA4* genes. As expected, these strains show the same phenotypes as the previously studied *VATA*, *VATB* and *VAT<u>c</u>* disruptants. We are currently attempting to precisely characterize the relative missorting phenotypes of ALP relative to the soluble hydrolases by generating conditional mutants in *VAT* genes. In addition, we are performing subunit depletion experiments with these gene products by placing them under the control of the repressible *GAL10* promoter. Both of these approaches should allow us to define the onset of the sorting defect and to further characterize the role of acidification in vacuolar protein sorting.

H 338 AMINOPEPTIDASE I IS LOCALIZED TO THE VACUOLE INDEPENDENT OF THE SECRETORY PATHWAY, Daniel J. Klionsky, Rosario Cueva, Debbie S. Yaver and Tanya M. Antes, Department of Microbiology, University of California, Davis, CA 95616

The vacuole of the yeast *Saccharomyces cerevisiae* is integrally involved in a variety of cellular functions. These include metabolite storage, ion homeostasis, osmoregulation and protein turnover. Accurate and efficient delivery of the resident vacuolar proteins is critical to the organelle's ability to carry out its role(s) in cellular metabolism. Most of the characterized vacuolar proteins transit to this organelle through the secretory pathway. They utilize an amino terminal signal peptide to enter the ER. Subsequently, these proteins are transported to the Golgi complex where they are sorted from other secretory pathway proteins and are directed to the vacuole.

The vacuolar hydrolase aminopeptidase I (API) was initially characterized as being a glycoprotein that was delivered to the vacuole via the secretory pathway. API lacks a standard hydrophobic signal sequence, however, suggesting that it would not be able to translocate into the ER. To address this apparent discrepancy, we undertook a careful immunological analysis of API. API is synthesized as a higher molecular weight precursor that is processed by protease B. The half-time for this processing event, however, is approximately 45 minutes--about 9 times longer than that seen for other soluble vacuolar hydrolases. We also found that API is not glycosylated based on the following: (1) It is unable to bind the lectin concanavalin A; (2) Its migration in an SDS gel is unaffected by tunicamycin; (3) The in vitro product migrates at the same position as the authentic in vivo precursor. In addition, the processing of API appears to occur independent of *SEC* proteins. Finally, API is not secreted from yeast cells upon overproduction or as a result of vacuolar protein sorting (*vps*) mutations that cause the missorting and secretion of other soluble vacuolar proteins.

These results suggest that the API precursor resides in the cytoplasm and is translocated directly into the vacuole. This is similar to the mechanism proposed for the vacuolar membrane protein α-mannosidase. We are using API as a marker protein to investigate this alternative mechanism of vacuolar localization, utilizing both in vitro and genetic analyses.

H 339 *SSV17* ENCODES A PROTEIN INVOLVED IN CORRECT VACUOLAR SEGREGATION., Caroline A. Hartley, Martin Latterich and Martin D. Watson, Department of Biological Sciences, University of Durham, Durham, DH1 3LE.

A series of mutations, termed *ssv*, have been isolated and characterised in *Saccharomyces cerevisiae* that confer sensitivity to osmotic stress [1]. Most of these also have a defect in vacuolar structure and/or protein sorting. These have been assigned to 17 genes of which only two have been identified before. One of these, *SSV17*, encodes a protein involved both in vacuolar protein sorting and segregation to the daughter bud. This has been characterised by indirect immunofluorescence using antibodies to two different vacuolar membrane proteins, Pho8p and the 60kDa ATPase subunit [2]. Within a population of cells can be found a heterogeneous mix with regard to vacuolar morphology. Some budding cells are completely normal and vacuoles in both mother and daughter cells can be seen. In other budding cells only the mother cell has a visible vacuole, even when the bud is quite large and nuclear segregation has occurred. Normally vacuolar segregation precedes nuclear segregation. Lastly budding cells can be observed in which both mother and daughter completely lack a vacuole.

The *SSV17* gene has been cloned. Characterisation of this gene is in progress.

1. Latterich, M. & Watson, M.D. *Mol. Microbiol.* 5, 2417-2426 (1991).
2. Roberts, C.J., Raymond, C.K., Yamashiro, C.T. & Stevens, T.H. *Meth. Enzymol.* **194**, 644-661 (1991).

H 340 *SSV7* ENCODES A PUTATIVE GUANINE NUCLEOTIDE EXCHANGE FACTOR INVOLVED IN NUCLEAR MEMBRANE ASSEMBLY, Martin D. Watson, Caroline A. Hartley and Martin Latterich, Department of Biological Sciences, University of Durham, Durham DH1 3LE, UK.

The *SSV7* gene of *Saccharomyces cerevisiae* encodes a protein of 927 amino acids with a predicted molecular mass of 105kDa. The protein consists of three functional domains. The C-terminal 300 amino acids show homology with guanine nucleotide exchange factors such as Cdc25p and Bud5p. The N-terminal 400 amino acids shows homology with gramicidin-S-synthetase I, probably a spurious relationship. The middle sequence forms a linking domain.

Mutations in *SSV7* result in sensitivity to osmotic stress, a mild vps phenotype and a non-lethal anuclear phenotype. The growth rate of *ssv7* cells is half that of wild-type but the cells are large being on average 2-3 times larger than normal haploid cells. Budding appears to be normal.

H 341 A TEMPERATURE-CONDITIONAL YEAST *VPS*18 MUTANT BLOCKS MEMBRANE PROTEIN TRAFFIC TO THE VACUOLE, Karl Köhrer, Tom A. Vida and Scott D. Emr, Div. of Cellular and Molecular Medicine, Howard Hughes Medical Institute, UCSD School of Medicine, La Jolla, CA 92093-0668.

The *vps*18 mutant was isolated in a selection for mutants that are defective in vacuolar protein sorting. The cloned *VPS*18 gene encodes a 110 kDa protein with a Cys-rich, zinc finger-like motif at the C-terminus. Changing Cys_{826} to Ser within this motif results in a temperature-conditional *vps*18 allele ($vps18^{ts}$). Analysis of the $vps18^{ts}$ allele demonstrated that vacuolar protein transport was normal at the permissive temperature (22°C). However, immediately upon shift to 37°C, precursor forms of both membrane-bound and soluble vacuolar proteins accumulated in an intracellular compartment. This transport block was reversible after cells were shifted back to the permissive temperature. Surprisingly, at a semi-permissive temperature (30°C), transport of the vacuolar membrane protein, alkaline phosphatase (ALP), was completely blocked while transport of the soluble vacuolar protein CPY was normal. These data suggest that independent transport carriers may participate in the delivery of membrane versus soluble proteins to the vacuole. Using antiserum against Vps18p, we demonstrated that the Vps18p was a relatively stable protein. Differential centrifugation showed that the Vps18p existed in two pools equally distributed between the cytosol and a particulate fraction sedimenting at 100,000 *x g*. The particulate fraction of the Vps18p was soluble in Triton X-100, suggesting that it was membrane-associated. In sucrose-density gradients, the protein cofractionates with a compartment distinct from the ER, late Golgi (Kex2p), and the vacuole, suggesting that it may associate with an intermediate in Golgi to vacuole transport (e.g. endosome).

Late Abstract

INTERACTION OF MUTATED PHASEOLIN POLYPEPTIDES WITH LUMENAL BINDING PROTEIN IN TOBACCO PROTOPLASTS. Aldo Ceriotti, Emanuela Pedrazzini, Giovanna Giovinazzo*, Roberto Bollini and Alessandro Vitale, Istituto Biosintesi Vegetali, Consiglio Nazionale delle Ricerche, Via Bassini 15, 20133 Milano, and (*)Istituto di Ricerca sulle Biotecnologie Agroalimentari, Lecce, Italy.

Phaseolin (PHSL) is a trimeric glycoprotein that is synthesized during seed development in bean (Phaseolus vulgaris) cotyledons. The nascent polypeptides are translocated into the lumen of the endoplasmic reticulum and assembled into trimers. Trimeric PHSL is then transported to the storage vacuoles of cotyledonary parenchima cells. We have recently shown that ER associated PHSL polypeptides interact with an ER resident protein that is the plant homolog of mammalian and yeast lumenal binding protein (BiP). To further investigate the role of BiP in the synthesis of PHSL we have expressed wild type and mutant PHSL polypeptides in tobacco protoplasts using a transient expression system. Wild type PHSL readily oligomerizes but is then degraded via the formation of discrete intermediates. No association of BiP with wild type PHSL could be detected in this system. Since the X-ray structure of PHSL is know, we were able to produce a set of mutants which are impaired in oligomerization. Mutated PHSL subunits are found in association with tobacco BiP and are then degraded without the formation of detectable intermediates. BiP can be released in vitro from these mutant polypeptides by ATP treatment. The site of degradation of wild type and mutant PHSL polypeptides is presently being investigated.