

THE MECHANISM OF PROTEIN IMPORT INTO CHLOROPLASTS AND ITS ROLE IN PLASTID DEVELOPMENT

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Chloroplasts represent one member of a diverse group of essential organelles referred to collectively as the plastids. All plastid types develop from a precursor organelle, the proplastid, in response to the particular metabolic demands of the differentiated cell type in which they function. Plastid differentiation relies on the expression and import of select classes of nuclear-encoded preproteins from their site of synthesis in the cytoplasm. Import is directed by interactions between the intrinsic N-terminal transit sequence of the preprotein and multisubunit complexes in the outer (Toc complex) and inner (Tic complex) plastid envelope membranes (1-3). We are using combined biochemical and reverse genetic approaches to investigate the roles of the Toc and Tic components in protein import and determine their possible roles in plastid differentiation.

Import consists of three stages: 1) transit sequence binding at the Toc complex, 2) ATP/GTP-dependent insertion of the preprotein across the outer envelope membrane, and 3) ATP-dependent insertion across the inner envelope membrane and simultaneous translocation across both envelope membranes (Fig. 1). The Toc complex performs the crucial functions of preprotein recognition and initiation of membrane translocation. Three outer envelope membrane proteins, Toc34, Toc75, and Toc159, form the central components of the Toc complex. Analysis of Toc159 function in pea (psToc159) using *in vitro* import assays indicates that Toc159 associates with preproteins at the early stages of protein import (4-6). These data have led to the proposal that Toc159 constitutes the major preprotein receptor at the chloroplast surface. To investigate the function of Toc159 *in vivo*, we sought to identify mutants in *Arabidopsis thaliana* *TOC159* using a reverse genetic approach. Searches of *Arabidopsis* genomic sequence databases revealed the existence of a family of three putative import receptor proteins related to psToc159 (Fig. 2). We designate these putative receptor proteins, atToc159, atToc132 and atToc120 on the basis of their deduced molecular masses in kilodaltons. AtToc159, -132, and -120 have a tripartite structure (Fig. 2). The GTP-binding (G-domains) and C-terminal membrane anchor domains (M-domains) are highly conserved among the three proteins (~65% identity) whereas the N-terminal acidic domains (A-domains) vary

considerably in sequence (~20% identity) and length. AtToc159 and psToc159 are most closely related (48% overall identity; 74% identity in the GTP-binding and C-terminal domains), suggesting that they are functional orthologs (7, 8).

We identified T-DNA insertion mutants in *Arabidopsis* that disrupt the *TOC159* gene (9). Remarkably, an atToc159 null mutant, designated *ppi2*, shows a specific defect in the import of light-induced photosynthetic proteins. The expression and import of constitutively-expressed plastid proteins is normal in *ppi2*. The import defect leads to a block in the differentiation of chloroplasts from proplastids, whereas other plastid types appear to develop normally. The characteristics of the *ppi2* mutant demonstrate a role for protein import as a limiting process in chloroplast biogenesis and reveal previously unknown alternative pathways for targeting proteins to plastids. The atToc159 defect limits the capacity of plastids to import a set of highly expressed photosynthetic proteins essential for chloroplast biogenesis. The atToc120 and atToc132 genes are expressed at different levels in different tissues, suggesting that they might account for residual import activity in the absence of atToc159. The functional differences among the import receptors are likely to involve the cytoplasmic A-domains where atToc159, -132 and -120 structurally are most divergent. These data support the hypothesis that plastids contain multiple import complexes that support the import of select, but overlapping sets of preprotein substrates. We will present our recent data on the role of the atToc159 receptor family in protein import. In addition, we will report on the in vivo analysis of additional Toc and Tic components.

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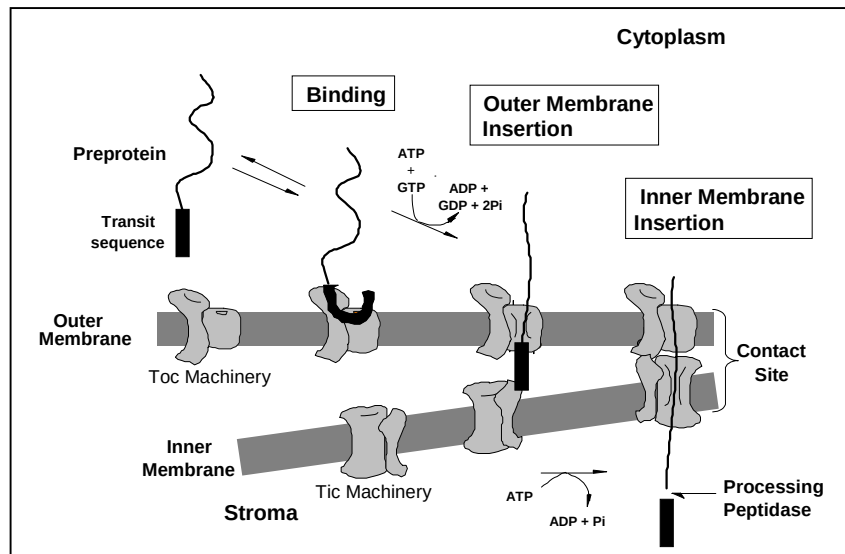


Figure 1. The stages of protein import into chloroplasts detected in in vitro import assays using isolated chloroplasts and recombinant preproteins.

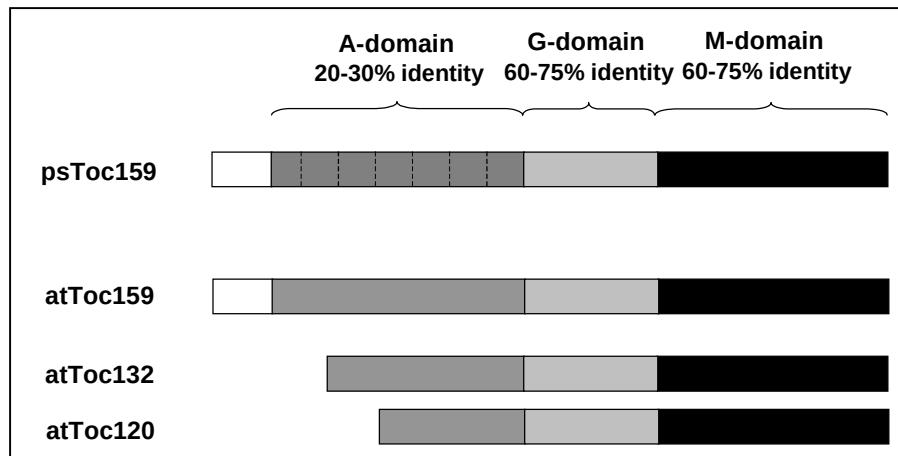


Figure 2. Schematic representation of the primary structures of psToc159, atToc159, atToc132, and atToc120. The diagrams are aligned to highlight maximum primary structure similarity. The positions of the repetitive, acidic domains (A domain), the GTP-binding domains (G domain), and the membrane anchor domains (M domain) and the approximate degree of amino acid sequence identity among the domains of the proteins are indicated at the top of the figure.

BIOCHEMICAL & GENETIC ANALYSIS OF LIPID TRANSPORT IN YEAST

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The topological segregation of the enzymes involved in the metabolism of phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) provides a powerful tool for the biochemical and genetic analysis of steps in the interorganelle movement of the aminoglycerophospholipids. In *Saccharomyces cerevisiae*, PtdSer is synthesized in the endoplasmic reticulum and closely related membranes and subsequently metabolized to PtdEtn by PtdSer decarboxylase 1 (Psd1p) in the mitochondria or Psd2p in the Golgi vacuole. The newly formed PtdEtn present in either organelle is exported out of these organelles and further metabolized to PtdCho in the ER. In strains harboring a *psd1Δ* allele all denovo aminoglycerophospholipid metabolism must transit through the Golgi/vacuole compartment. Using *psd1Δ* strains we have screened for new strains defective in PtdSer transport (*pstB*) and PtdEtn export (*peeB*) by isolating ethanolamine (Etn) auxotrophs. We have identified two new mutants, *pstB2* and *peeB1* that are defective in the metabolism of nascent PtdSer and PtdEtn, respectively. Biochemical and genetic analyses reveal that neither mutant has defects in PtdSer synthase, PtdSer decarboxylase 2 or PtdEtn, methyltransferases. The *pstB2* mutant accumulates PtdSer and is defective in the conversion to PtdEtn. The PtdSer accumulates in the Golgi and a novel light membrane fraction in permeabilized cells. The gene that complements the defect, *PSTB2* encodes a non-essential homolog of Sec14p a PtdIns/PtdCho transfer protein. The PstB2p is capable of transferring PtdIns but not PtdCho or PtdSer. Strikingly, the *PSTB2* gene is a high copy suppressor of *SEC14*, but high copy *SEC14* gene will not suppress *pstB2* mutants. The *peeB1* mutant is defective in PtdCho formation and exhibits modest accumulation of PtdEtn and PtdSer. The *peeB1* mutant is complemented by *VAM6* encoding a protein involved in regulating vacuolar morphogenesis and protein sorting. Strains with *vam6Δ* alleles are Etn auxotrophs at 36° and show defects in PtdCho formation. Protein interaction studies have previously identified Vam2p as a protein that binds Vam6p generation of *vam2Δ* mutants also demonstrates that these strains are Etn auxotrophs at 36°C. Our studies are now revealing increasing

amounts of genetic information that provide the raw material for examining the biochemistry of interorganelle lipid transport. Supported by NIH GM32453.

THE DYNAMIN FAMILY OF MECHANOENZYMES: PINCHING IN NEW PLACES

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Dynamin has been implicated in the liberation of nascent vesicles from the plasma membrane during clathrin-mediated endocytosis. To test if the dynamin family participates in additional membrane trafficking events in mammalian epithelial cells, we have combined conventional biochemical and morphological methods with antibody microinjection and the expression of different GFP-tagged dynamin constructs. Microinjection of purified dynamin antibodies inhibited the clathrin-mediated endocytosis of fluorophore-labeled transferrin and caused a marked invagination of the plasma membrane. Many of these long plasmalemmal invaginations had clathrin-coated pits along their cytoplasmic surface. A number of distinct non-coated pits resembling plasmalemmal caveolae also accumulated in anti-dynamin antibody-injected cells. Further, the cellular uptake of cholera toxin B, which is normally internalized by caveolae, was inhibited in these cells. In support of these observations, immunoisolation techniques, double-label immunofluorescence microscopy and immuno-electron microscopy provided biochemical and morphological evidence that dynamin associates with plasmalemmal caveolae. Together, these observations indicate that dynamin mediates scission from the plasma membrane of both clathrin-coated pits and caveolae during distinct endocytic processes. Additional studies using GFP-tagged dynamin isoforms demonstrate that the different dynamin isoforms have a differential distribution in mammalian cells. Targeting information for these isoforms may be provided, at least in part, by regions of alternative splicing. Computer generated movies of GFP-dynamin in living cells, shown during the presentation, suggest that dynamin associates with both clathrin and AP2 at the cell surface to form large tubular-vesicular “plaques”. These structures are invaginations of the plasma membrane and internalize both fluid markers and transferrin while generating numerous motile, dynamin-associated vesicles. These studies suggest that dynamin and other coat proteins are clustered into endocytic “hotspots” along the ventral plasma membrane of living cells to generate large numbers of motile endocytic vesicles which translocate into the cytoplasm. In addition, these findings indicate that dynamin participates in multiple endocytic processes.

BIOPHYSICAL DISSECTION OF MEMBRANE AND PROTEIN

TRAFFIC AT SYNAPTIC NERVE TERMINALS. Timothy A. Ryan^{*} and

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Presynaptic nerve terminals recycle synaptic vesicle components through a local endocytic pathway. This pathway is critical, since suspension of recycling depletes presynaptic terminals of vesicles and they quickly fail to transmit information. The goal of our work is to determine what processes in the flow of membrane traffic are rate limiting during sustained synaptic activity, and in turn dissect the molecular basis for these processes. Over the last several years we have applied optical approaches for studying vesicle membrane recycling in synaptic terminals.

Recently we adopted a new approach that allows one to follow the fate of specific proteins during membrane recycling in response to action potential firing in the presynaptic terminal. We made use of new pH-sensitive variants of green fluorescent protein (GFP) whose pH-sensitivity is ideally matched to the detection of the transition from the acidic lumen of the synaptic vesicle (pH~ 5.5) to the more alkaline environment of the synaptic surface (pH ~7.4). Synaptic vesicles are specialized endosomes that maintain an acidic lumen due the activity of a vacuolar H⁺ ATPase. This activity is required to establish an electromotive force that in turn drives neurotransmitter uptake from the cytosol into the vesicle. Almost all GFPs are excellent pH sensors. Recent measurements of the pH-dependence of EGFP indicate that the fluorescence is completely quenched upon protonation at Tyr-66 (1). Thus the protonation reaction provides an all or none fluorescence switch. The pK of the protonation reaction determines what fraction of the GFPs is deprotonated at any given pH. These new GFP's, developed by James Rothman (2) and colleagues were termed pHluorins. pHluorins targeted to the synaptic vesicle lumen (synapto-pHluorin) enabled measurements of dynamic changes in pH of vesicle lumen

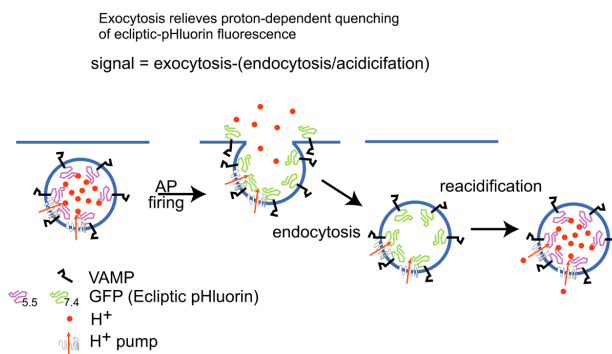


Figure 1. pH-based monitoring of synaptic vesicle protein recycling using synapto-pHluorin.

resulting from exocytosis and endocytosis of synaptic vesicles during pre-synaptic activity. The net fluorescence change observed during action potentials therefore reflect a net balance of externalized pHluorin, which is brightly fluorescent, and the endocytosed and reacidified vesicles, which are dark (Figure 2). We recently determined the pK of pHluorin to be ~ 7.1 which makes them well suited for studying the cycle of alkalization and reacidification that occurs during synaptic vesicle recycling.

Hippocampal neurons grown in culture were transfected with cDNA encoding a C-terminal fusion construct between the synaptic vesicle protein VAMP and pHluorin such that pHluorin becomes targeted to the synaptic vesicle lumen. We established that this technology, in combination with quantitative fluorescence microscopy, could be used to give real time measurements of the recycling of synaptic vesicle proteins. Using surface quenching techniques, we determined that the reacidification step following endocytosis is very fast (~ 1 sec) and therefore the fluorescence signal decay after the action potential firing stops is primarily limited by the kinetics of endocytosis (3). This system thus provides a robust, quantitative assay for studying the endocytosis of synaptic vesicle constituents following action potential firing at synaptic terminals. During this presentation, I will discuss new results derived from measurements using this approach. In particular we are concentrating our efforts on understanding how the speed and efficiency of endocytosis is modulated at synaptic terminals.

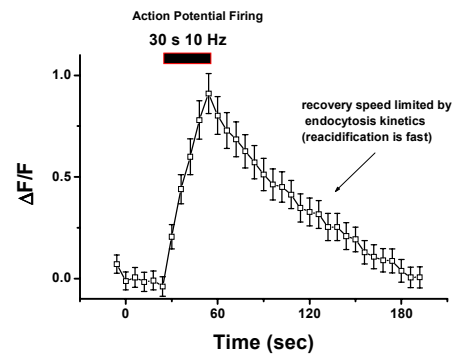


Figure 2. Synapto-pHluorin fluorescence during action potential firing. Ensemble average from ~ 20 synaptic boutons is displayed.

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TRANSFER OF THE RIBOSOME-NASCENT CHAIN COMPLEX FROM SRP TO THE SEC61 COMPLEX.

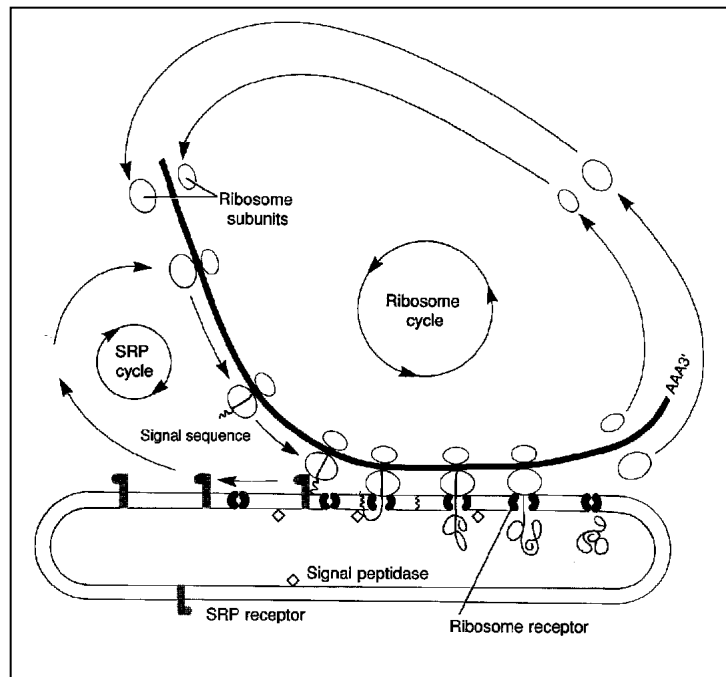
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Protein translocation across the rough endoplasmic reticulum is mediated by the signal recognition particle (SRP), the SRP receptor (SR), the TRAM protein and the Sec61 complex. The SRP binds to the N-terminal signal sequence of a nascent polypeptide as the protein is synthesized by the ribosome. The SRP-ribosome-nascent chain (RNC) complex is targeted to the RER via the interaction between SRP and the membrane-bound SRP receptor. Transfer of the SRP-RNC to the Sec61 complex is dependent upon GTP. The signal sequence binding subunit of SRP (SRP54) and both the alpha and beta subunits of the SRP receptor are members of the GTPase superfamily. Cooperative binding of GTP to both the alpha subunit of the SR and to SRP54 is required to initiate transfer of the signal sequence from SRP54 to the Sec61 complex (Rapiejko, P. and Gilmore, R. 1997. *Cell* 89:703-713.) A combination of biochemical and molecular biological approaches are being used to elucidate the roles of the SRP and SR GTPases. A novel translocation intermediate accumulates when the alpha subunit of the Sec61 complex is severed by proteases in cytoplasmic loop 6 or 8. In the absence of a functional Sec61 complex, the SRP-RNC complex is targeted to the SR. GTP binding to SRP54 and the alpha subunit of the SR was shown to stabilize the membrane bound SRP-RNC. A similar post-targeting intermediate accumulates when SRP-RNCs are targeted to SR-proteoliposomes. In both cases, GTP binding to the SRP-SR complex does not promote signal sequence dissociation from SRP54. These observations indicate that the Sec61 complex regulates the GTP hydrolysis cycle of the SRP-SR complex at the stage of signal dissociation from SRP54. The GTP binding site in the SR appears to be required for the assembly of the SR heterodimer and for the protein translocation reaction. Formation of SR heterodimers was evaluated using SR-beta and SR-alpha mutants with altered GTP binding sites. To determine whether the GTP binding site of SR-beta is required for protein translocation we conducted assays using SRP54 and SR-alpha mutants that bind xanthosine triphosphate (XTP) in preference to GTP. Using this experimental system, we observe that both XTP and GTP are required for transfer of a RNC from the SRP to the Sec61 complex.

THE REGULATION OF RIBOSOME EXCHANGE ON THE ENDOPLASMIC RETICULUM MEMBRANE.

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In current models of protein translocation in the endoplasmic reticulum (ER), ribosome exchange on the ER membrane serves a principal regulatory function. Thus, ribosome binding to the ER membrane elicits assembly of the translocon, the cohort of proteins that mediate the translocation and early covalent processing of nascent secretory and integral membrane proteins. Subsequently, the termination of protein synthesis yields the dissociation of the ribosomal subunits from the membrane and the coincident inactivation of translocon function. The cycle of ribosome exchange (illustrated below) is, therefore, an integral element of the molecular mechanism of protein translocation in the ER. To study the regulation of ribosome exchange on the ER membrane, two experimental approaches were utilized. In one approach, morphometric and biochemical analyses of ribosome-membrane interactions during the protein translation cycle were examined. These experiments were performed in tissue culture cells and examined the state of membrane association following inhibitor based inhibition of elongation (cycloheximide), initiation (pactamycin), or induction of premature chain termination (puromycin). Run-off translation (pactamycin) or premature termination (puromycin treatment) was accompanied by the enhanced release of small ribosomal subunits from the ER membrane. Under these conditions, the majority of the large subunits remained in stable association with the ER membrane. Using *in vitro* systems, we observed that following run-off translation and re-isolation of microsomal membranes, ribosomal subunits remained in stable association with the ER membrane. Membrane-bound ribosomal subunits remaining after run-off translation are capable of initiating protein translation, regardless of whether the mRNA encoded a secretory protein or a soluble, cytoplasmic protein. Membrane-bound ribosomes engaged in the synthesis of soluble, cytoplasmic proteins dissociate during the elongation cycle as intact ribosome/nascent chain complexes. This dissociation reaction thus maintains the compartmental segregation of secretory and cytoplasmic protein synthesis. We conclude that coincident with the termination of protein synthesis, membrane-bound small ribosomal subunits become available for the initiation stage of protein synthesis and large ribosomal subunits remain in stable association with the ER membrane. We propose that physiological ribosome release from the ER membrane requires that the membrane bound ribosome engage in the synthesis of a protein lacking a signal sequence or transmembrane domain.



Schematic Illustration of the Ribosome Cycle.

Following initiation of protein translation on free, soluble ribosomes, nascent chains bearing a signal sequence are targeted in an SRP-dependent manner to the endoplasmic reticulum membrane. Translation and translocation then proceeds, yielding transfer of the nascent chain into the ER lumen, or integration into the ER membrane. Following the termination of protein translation, ribosomal subunits release from the membrane and re-enter the cytoplasmic pool.

MECHANISM OF PROTEIN IMPORT INTO THE PEROXISOMAL MATRIX AND MEMBRANE.

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Like the sorting of proteins into other sub-cellular compartments, the targeting of proteins to the peroxisomal matrix and membrane is facilitated by the use of specific targeting signals that act in concert with proteins comprising the peroxisome-specific import machineries. Two pathways exist for the targeting of proteins to the peroxisomal matrix, and these use well-defined tripeptide or nonapeptide peroxisomal targeting signals (PTSs), called PTS1 and PTS2, that reside either at the carboxyl- or amino termini of cargo proteins. Peroxisomal membrane proteins (PMPs) use distinct mPTSs for their sorting to the peroxisomal membrane.

The PTS1 and PTS2 sequences are recognized by cytosolic receptors, Pex5p and Pex7p, respectively, that shuttle the cargo to peroxisome membrane-associated proteins, Pex13p and Pex14p. Other peroxins, Pex17p and Pex3p, are also components of this matrix-protein import machinery. Following the docking of the receptor-cargo complex with the matrix-protein import machinery, the cargo alone, or the receptor-cargo complex, is translocated across the membrane by an unknown mechanism.

While much progress has been made in our understanding of matrix-protein import, we have only recently begun to make a dent in PMP import. At least four peroxins, Pex3p, Pex16p, Pex17p and Pex19p play a role in the targeting and/or assembly of PMPs. Multiple PMPs interact with the peroxin, Pex19p, which is primarily cytosolic and partially peroxisomal. The integral PMPs, Pex3p and Pex17p, are components of both the matrix- and membrane-protein import machineries, forming distinct complexes. The phenotypes of cells lacking these peroxins, the knowledge of the interactions between these proteins, and the recognition that they form distinct sub-complexes, suggests models for the functions of these proteins in PMP import.

A VESICULAR PATHWAY FROM THE GOLGI DEPENDS ON GLYCOLIPIDS. ROLE FOR THE MULTIDRUG TRANSPORTER MDR1 P-GLYCOPROTEIN AS A GLYCOLIPID FLIPPASE

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In mammalian cells most glycolipids have a ceramide backbone and are consequently termed glycosphingolipids. While the content of glycosphingolipids is generally low in intracellular membranes, they are enriched on the cell surface. Glycosphingolipids constitute from a few percent in the outer leaflet of the plasma membrane in fibroblasts to virtually 100% of the polar lipids in the outer leaflet of the apical plasma membrane domain of intestinal epithelial cells. The simplest glycosphingolipids are galactosylceramide and glucosylceramide. Galactosylceramide is expressed in specific cells, especially in cells that synthesize myelin. Knock-out mice for the ceramide galactosyltransferase, which synthesizes galactosylceramide, display severe nerve pathologies resulting in paralysis and death due to myelin malfunction (1, 2). Glucosylceramide is found in every animal cell. It is the precursor for essentially all complex glycolipids. Knock-out mice for the ceramide glucosyltransferase die as embryos (3). This most likely reflects a function for the complex glycolipids in cell-cell and cell-substratum recognition and differentiation. Remarkably, individual cells without glycolipids grow and proliferate (3, 4). Glucosylceramide is synthesized on the cytosolic surface of the Golgi apparatus. Part of it is translocated across the Golgi membrane to the luminal leaflet where it can be converted to lactosylceramide and higher glycolipids. However, in fibroblasts some 50% does not cross the Golgi membrane but is transported to the cytosolic surface of the plasma membrane. From there it reaches a site where it is degraded by an enzyme that has previously been characterized as a non-lysosomal glucosylceramidase (5). This hydrolysis is prevented by the activity of the multidrug transporter MDR1 P-glycoprotein (6). We have found that this protein is capable of translocating short-chain GlcCer from the cytoplasmic leaflet into the exoplasmic leaflet of the plasma membrane (7). We have now demonstrated by using an enzymatic assay that MDR1 P-glycoprotein translocates natural glucosylceramide across the plasma membrane (6). In addition, we report a function for glucosylceramide in the sorting of transmembrane proteins out of the Golgi into the AP-3 pathway, a vesicular pathway towards specialized organelles like melanosomes (8). For these studies we have made use of a cell line unable to synthesize glycolipids (4) and of inhibitors of various enzymes of glucosylceramide metabolism (9, 10). The data suggest a sorting function for glucosylceramide at the cytoplasmic surface of the Golgi, and regulation of this function by multidrug transporters and hydrolytic enzymes. Possible molecular mechanisms will be discussed.

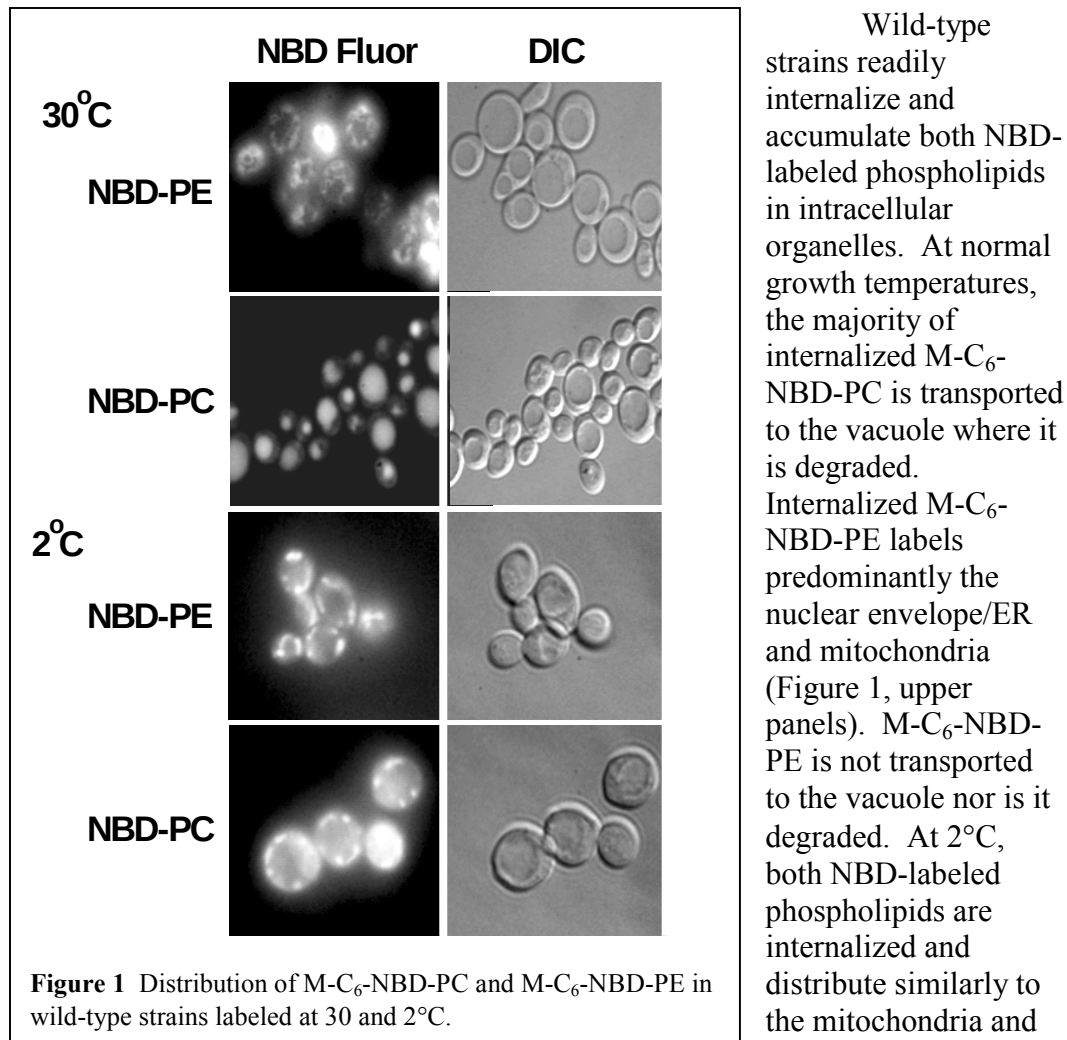
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PHOSPHOLIPID TRAFFICKING IN YEAST

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Eukaryotic cells ranging from yeasts to mammals maintain non-random, non-equilibrium distributions of phospholipids among and across their membranous organelles. The molecular details of how these asymmetric membrane distributions are established and maintained as well as their physiological significance are poorly understood. To address these questions, the internalization and distribution of phosphatidylcholine and phosphatidylethanolamine was monitored using fluorescent, NBD-labeled analogues, M-C₆-NBD-PC and M-C₆-NBD-PE, respectively, in the genetically tractable yeast, *S. cerevisiae*.



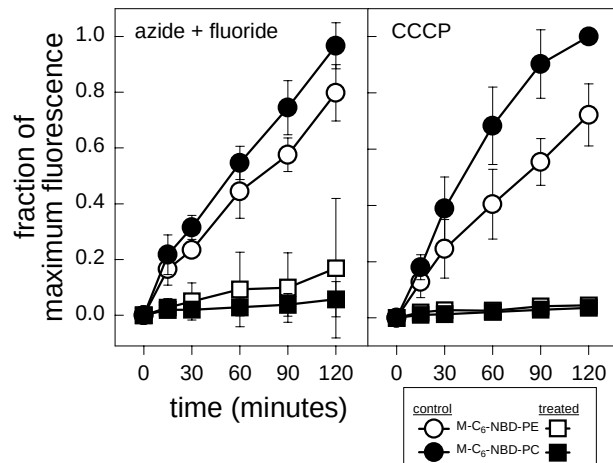


Figure 2: Low temperature internalization of M-C₆-NBD-PC and M-C₆-NBD-PE measured by flow cytometry.

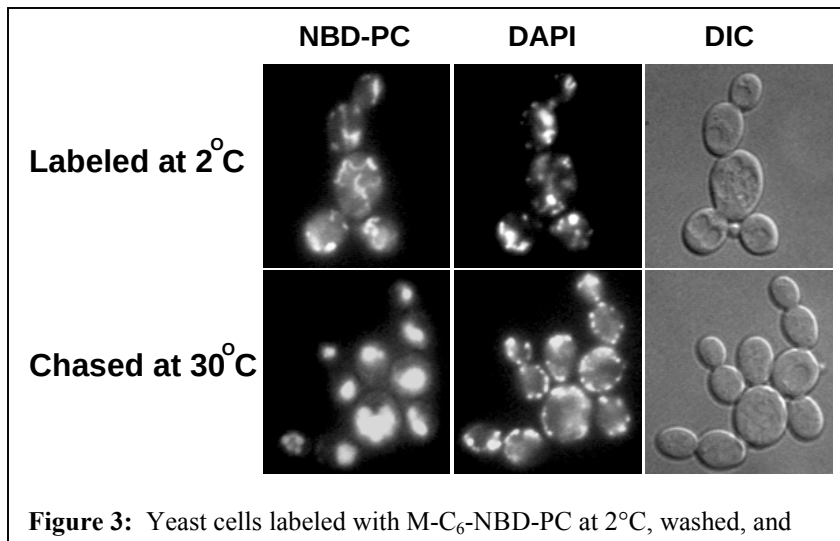
washing, the fluorescence intensity of each cell reflects only internalized NBD. Figure 2 illustrates that M-C₆-NBD-PC and M-C₆-NBD-PE are internalized at roughly the same rate at 2°C and that the internalization of both is dramatically inhibited by energy depletion by pretreatment with azide and fluoride or by collapse of the proton electrochemical potential with the protonophore, CCCP. Given that endocytosis and vesicular transport are blocked at 2°C, these data suggest that at low temperature M-C₆-NBD-PC and M-C₆-NBD-PE are translocated across the plasma membrane by an energy-dependent transporter. Furthermore, the internalization of neither M-C₆-NBD-PC nor M-C₆-NBD-PE is inhibited at the non-permissive temperature in endocytosis mutants, *end4-1* and *act1-1*, suggesting that transbilayer transport, not endocytosis, is the predominant mechanism of uptake for both phospholipids at normal growth temperatures as well.

If this conclusion is true, M-C₆-NBD-PC is predicted to be trafficked to the lumen of the vacuole, not by endocytosis from the plasma membrane, but from the cytosolic topological compartment. To test this prediction, the mitochondria and nuclear envelope/ER of a wild-type strain were labeled with M-C₆-NBD-PC at 2°C, washed, and allowed to incubate at room temperature. The result (Figure 3) demonstrates that M-C₆-NBD-PC is trafficked to the vacuole subsequent to its internalization to the cytosolic compartment. In an analogous experiment, M-C₆-NBD-PE was not trafficked to the vacuole.

To determine whether M-C₆-NBD-PC traffic to the lumen of the vacuole is mediated by the vacuolar protein sorting pathway, M-C₆-NBD-PC

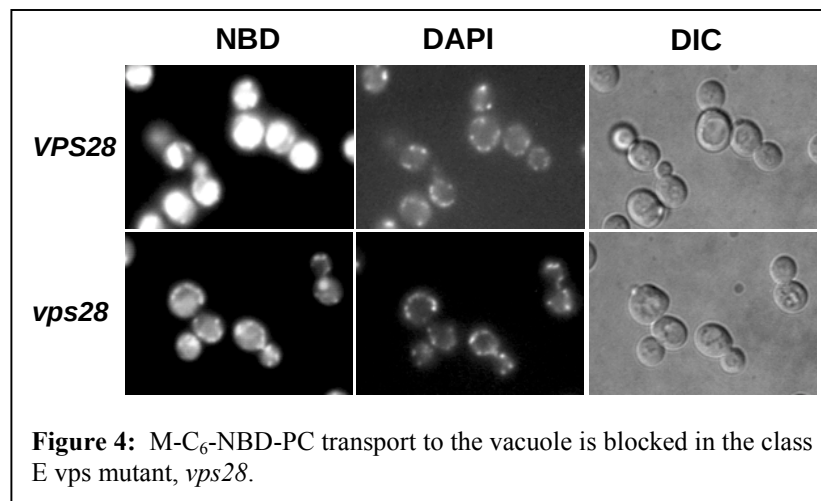
nuclear envelope/ER (Figure 1, lower panels). M-C₆-NBD-PC transport to and degradation in the vacuole is blocked.

Flow cytometry provides a convenient method for quantifying the internalization of M-C₆-NBD-PC and M-C₆-NBD-PE. Since no detectable NBD fluorescence remains associated with the cell surface following



internalization was tested in the class E *vps* mutant, *vps28*. The results (Figure 4) illustrate that M-C₆-NBD-PC traffic to the vacuole is inhibited in *vps28* suggesting that its transport to the vacuole is dependent on vesicular traffic from the prevacuolar compartment to the vacuole.

Based on these experimental results, we propose that M-C₆-NBD-PC and M-C₆-NBD-PE are predominantly transported from the outer to inner leaflet of the plasma membrane by similar, energy-dependent, proton electrochemical gradient-dependent transporters. Following translocation to the inner leaflet of the plasma membrane, both spontaneously redistribute to the mitochondria and nuclear envelope/ER. At this point, M-C₆-NBD-PE and M-C₆-NBD-PC are independently sorted such that only M-C₆-NBD-PC is transported through the prevacuolar compartment to the lumen of the vacuole.



LIPID TRANSPORT IN THE YEAST

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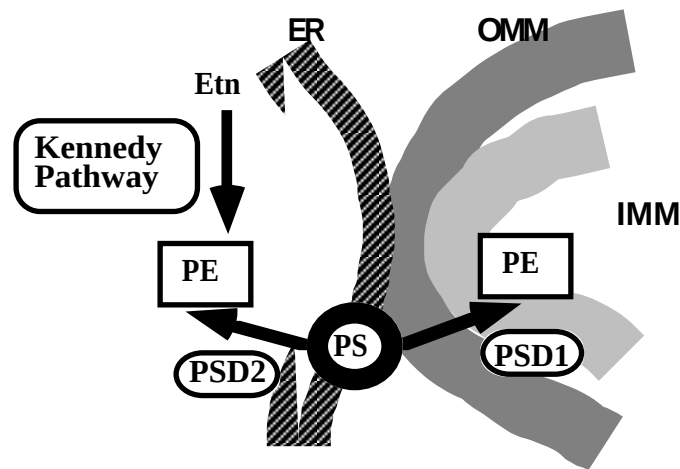
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The yeast *Saccharomyces cerevisiae* is a suitable system to study cell biological and molecular biological problems of organelle biogenesis. Synthesis and correct distribution of lipids are essential prerequisites for the assembly of functional membranes. Many lipids such as the major classes of phospholipids are distributed among all yeast subcellular membranes. Some lipids, however, are highly concentrated in specific organelles, such as sterols in the plasma membrane, cardiolipin in mitochondria, or triacylglycerols and sterol esters in lipid particles. The majority of enzymes involved in lipid biosynthesis is located in the endoplasmic reticulum. In addition, certain steps of lipid synthesis occur in mitochondria, Golgi and lipid particles. Interplay of these organelles is required to maintain a balanced cellular level of lipids. A prominent example of organelle collaboration is the sequence of biosynthetic steps of aminoglycerophospholipid synthesis, which occur in a concerted action of the endoplasmic reticulum, mitochondria and most likely the Golgi.

Intracellular transport of lipids is necessary for the supply of lipids to membranes that are unable to synthesize their own lipids, and the flux of intermediates between organelles that collaborate in lipid biosynthetic pathways. Protein-catalyzed lipid transport, vesicle flux and lipid translocation via membrane contact are possible mechanisms of lipid migration within the cell. Experimental evidence suggests that vesicle flow and contact between organelles are most likely the predominant mechanisms of lipid transport between subcellular compartments.

The plasma membrane of *Saccharomyces cerevisiae* lacks the capacity to synthesize its own lipids. Thus, efficient transport mechanisms must exist for the migration of these components from their site of synthesis to the cell periphery. While there is good evidence that sphingolipids of the yeast reach the plasma membrane through the protein secretory pathway less information is available for the transport of phospholipids and sterols to the yeast plasma membrane. To address the question as to the involvement of the protein secretory pathway in lipid translocation to the plasma membrane, we designed pulse-chase experiments with subsequent isolation of the plasma membrane to measure the amount of radioactively labeled lipid translocated to the cell periphery. These experiments were carried out with different temperature-sensitive secretory mutants conditionally blocked at different stages of the secretory pathway. Our results demonstrated that phosphatidylserine (PtdSer) and ergosterol follow the protein secretory route on their way to the cell periphery. In contrast, phosphatidylethanolamine (PtdEtn) and phosphatidylinositol (PtdIns) are transported to the plasma membrane by other mechanisms. Membrane contact of lipid-synthesizing membranes, especially the endoplasmic reticulum, with the plasma membrane may be considered as an alternative.

A major route of lipid transport is the import into mitochondria. It is not only the supply of PtdSer to the major site of PtdSer decarboxylation in the inner mitochondrial membrane that is important, but also the transport of extramitochondrially synthesized PtdEtn and PtdCho to their mitochondrial destination.



Three biosynthetic branches lead to the formation of PtdEtn, namely decarboxylation of PtdSer by (i) Psd1p in mitochondria (ii) Psd2p in a Golgi/vacuolar compartment, and (iii) the CDP-Etn pathway (Kennedy pathway). The contribution of each pathway to formation of mitochondrial PtdEtn was tested in double labeling experiments with [^3H]serine and [^{14}C]ethanolamine in strains deleted of *PSD1* or *PSD2*, respectively. The majority of mitochondrial PtdEtn is formed by Psd1p, but deletion of *PSD2* led also to a decrease of decarboxylation-derived PtdEtn as compared to the Kennedy pathway. Although supply of mitochondria with extramitochondrially synthesized PtdEtn through Psd2p or the Kennedy pathway is less efficient, import of PtdEtn into mitochondria significantly contributes to the maintenance of the mitochondrial PtdEtn level.

Strains defective in genes encoding PtdSer synthase (*cho1*) or both PtdSer decarboxylases (*psd1 psd2*) can be rescued either by ethanolamine (Etn) and/or choline (Cho) supplementation on glucose medium. Etn and Cho are utilized via the Kennedy pathway for PtdEtn and PtdCho synthesis. In addition to decarboxylation and Kennedy pathway, however, incorporation of endogenous ethanolaminephosphate (Etn-P) produced by sphingosine-phosphate lyase Dpl1p-dependent sphingolipid turnover contributes to a balanced level of PtdEtn. When Dpl1p is overexpressed in *cho1* or *psd1 psd2* background, strains are no longer auxotrophic for Etn or Cho. Blocked PtdSer decarboxylation and sphingolipid turnover in a *psd1 psd2 dpl1* triple mutant, on the other hand, results in strict Etn auxotrophy. Since this triple mutant cannot be rescued by Cho we conclude that PtdEtn is essential. The requirement of a minimum level of mitochondrial PtdEtn for respiration is documented best by the inability of *psd1* or *cho1* to grow on non-fermentable carbon sources in minimal media without Etn supplementation.

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COPII Dependent Transport between the ER and Golgi Complex in Yeast

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My research group is interested in molecular mechanisms of intracellular transport and we seek to understand how proteins catalyze distinct sub-reactions in traffic between the ER and Golgi. Cell-free transport of a labeled secretory protein from the ER to Golgi membranes has been reproduced with washed membranes, purified proteins and energy. Budding from the ER requires a set of coat proteins (Sar1p, Sec23p complex and Sec13p complex) that generate diffusible transport intermediates, termed COPII coated vesicles. Once these vesicles have uncoated, fusion with the Golgi complex proceeds in two biochemically distinct steps. First, vesicles are "tethered" to acceptor membranes in a reaction that requires Uso1p, Ypt1p and the Sec34p/Sec35p complex. Second, fusion of tethered vesicles depends on Sec18p, LMA1, Sly1p and the SNARE proteins Sed5p, Bet1p and Bos1p.

We have also identified and characterized a set of membrane bound ER-vesicle proteins (Erv proteins) that participate in transport between the ER and Golgi. Several of these proteins corresponded to uncharacterized open reading frames in the yeast genome and are under investigation. Genetic experiments indicate Erv14p, Erv25p, Erv41p and Erv46p function in protein sorting during transport between the ER and Golgi. Specific antibodies prepared against these Erv proteins demonstrate an ER/Golgi localization pattern and in vitro budding assays indicate these proteins are indeed selectively packaged into COPII vesicles. Yeast strains lacking Erv14p or Erv25p display defects in the transport of some secretory proteins from the ER whereas other secretory cargo are transported at wild-type rates. We propose that these Erv proteins function with the COPII coat in the selection of secretory cargo during transport vesicle budding from the ER. Other uncharacterized Erv proteins appear to participate in the vesicle fusion stage of this transport reaction. We continue to explore the mechanisms that underlie protein sorting and vesicle fusion using this refined system.

ER TO GOLGI TRANSPORT OF GPI-ANCHORED PROTEINS

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GPI-anchored protein transport from the ER to the Golgi compartment requires a functional ceramide synthesis pathway. This has been shown by experiments using either an inhibitor¹ of serine palmitoyltransferase (SPT) or a temperature-sensitive mutant² in the enzyme. When SPT is inactivated GPI-anchored protein transport to the Golgi compartment is greatly reduced, but transport of other secretory proteins is unaffected. To explain this selective transport defect we postulated that GPI-anchored proteins are transported from ER to the Golgi apparatus in vesicles that are distinct from those carrying other cargo molecules, such as transmembrane proteins. To test this we reproduced a single round of vesicle budding from the ER *in vitro* and analyzed the vesicles using immunoisolation techniques. A GPI-anchored protein, Gas1p, is clearly incorporated into different vesicles than the general amino acid permease, Gap1p, or the pheromone precursor, proalpha factor. We are currently performing studies that should aid in determining which lipid in the ceramide/sphingolipid synthesis pathway needs to be made for efficient GPI-anchored protein transport. Our results show that protein sorting takes place earlier in the secretory pathway than previously thought, in the endoplasmic reticulum and suggest that lipids could play a role in this process.

Another specific requirement for the transport of Gas1p is for members of the Emp24 family. This family consists of type I transmembrane proteins of approximately 24 kDa, with short cytosolic tails. Emp24p forms a protein complex with Emp25p⁴, Erp1p, and Erp2p⁵. Deletion of the corresponding genes cause a selective transport defect from ER to the Golgi compartment making them candidates to be receptors involved in cargo recruitment in the ER³. We tested this hypothesis using the *in vitro* budding assay. *emp24Δ* mutant membranes show a budding defect. Budding of Gas1p was strongly and selectively inhibited. Wild type membranes incubated with antibodies against the cytoplasmic tail of Emp24p behaved like *emp24Δ* mutant membranes. These results show that Emp24p specifically and directly enhances Gas1p budding. It is unlikely that this is due to a general budding defect because budding of proalpha factor is unaffected. Unlike Gas1p, Emp24p seems to be present in all ER-derived vesicles, thus its loss probably does not affect budding of a specific vesicle population. This suggests that Emp24p is involved in the specific recruitment of Gas1p into ER-derived vesicles. Consistent with this, Gas1p could be specifically and directly cross-linked to Emp24p and Erv25p in ER-derived vesicles, consistent the

receptor hypothesis⁶. These results also provide support for protein sorting in the ER.

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BIOGENESIS OF COPI-VESICLES

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COPI-vesicles mediate transport within the early secretory pathway. The following features of their formation will be described:

- Their cytosolic coat proteins ARF and coatamer are recruited dependent of the presence of GTP.
- Assembly of the machinery for the budding of a COPI vesicles involves a direct interaction of Coatamer with ARF-GTP^(1,2), and with Golgi-resident transmembrane proteins, whose cytosolic domain serves as a coatamer receptor⁽³⁾.
- Binding of coatamer to the receptor domains is via its γ -subunit (γ -COP)⁽⁴⁾ and this binding
- triggers a conformational change of coatamer, thereby inducing polymerization of the coat protein, a reaction that is likely to drive the formation of a curvature from the donor membrane⁽⁵⁾.
- This minimal machinery for budding was assembled functionally on chemically defined liposomes, as COPI-coated vesicles were efficiently formed in this reconstituted system⁽⁶⁾.
- Uptake of cargo into COPI-vesicles depends on the hydrolysis of GTP⁽⁷⁾ by ARF⁽⁸⁾.

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THE ROLE OF TWO RHO GTPASES IN COORDINATION OF
EXOCYTOSIS AND CELL POLARITY IN YEAST. Joan E. Adamo¹,
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Budding yeast grow asymmetrically by polarized delivery of proteins and lipids to specific sites on the plasma membrane. This requires the coordinated polarization of the actin cytoskeleton and the secretory apparatus. We identified Rho3 based on its genetic interactions with several late acting secretory genes. Mutational analysis of the Rho3 effector domain reveals three distinct functions in cell polarity: regulation of actin polarity, transport of exocytic vesicles from the mother cell to the bud, and docking and fusion of vesicles with the plasma membrane. Both electron microscopy and 2-hybrid analysis provide data which are consistent with the notion that the vesicle delivery function of Rho3 is mediated by the unconventional myosin, Myo2, and that the docking and fusion function is mediated by the exocyst component, Exo70. These data suggest that Rho3 acts as a key regulator of cell polarity and exocytosis, coordinating several distinct events for delivery of proteins to specific sites on the cell surface.

We have recently found evidence implicating another Rho GTPase, Cdc42, in regulation of the exocytic machinery at the cell surface. Using a novel temperature sensitive mutation, *cdc42-6*, we have found it to be readily suppressed by the same secretory components which we have previously shown suppress *rho3-V51*. In addition, genetic interactions between *rho3-V51* and *cdc42-6* strongly suggest that they have partially overlapping functions. Phenotypic analysis of *cdc42-6* shows a significant secretory defect for a distinct class of secreted proteins. This is accompanied by a pronounced accumulation of 80-100nm post-Golgi vesicles at the restrictive temperature. Interestingly, the vesicle accumulation defect in *cdc42-6* appears to be quite specific to a distinct stage of the cell cycle; as only small budded cells accumulated vesicles. In

contrast, when we examined the vesicle accumulation of *rho3-V51* cells, we found accumulation in both small and large budded cells. Taken together our results suggest a simple model by which the polarity of the actin cytoskeleton and the polarity of the exocytic machinery are coordinated at distinct stages of the cell cycle by the sequential action of these two Rho GTPases.

Membrane Traffic Along the Endocytic Pathway in Sphingolipid Storage Diseases.

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We are studying membrane lipid transport and sphingolipid/cholesterol interactions in sphingolipid storage disease (SLSD) fibroblasts. Recently we found that several fluorescent SL analogs were internalized from the plasma membrane predominantly to the Golgi complex of normal cells, while in 10 different SLSD cell types, these lipids accumulated in endosomes and lysosomes [The Lancet 354 (1999) 901-905]. This accumulation was in general unrelated to the extent of SL analog degradation in the different cell types. We also showed that cholesterol homeostasis is perturbed in multiple SLSDs secondary to SL accumulation and that mistargeting of SL analogs was regulated by cholesterol [Nature Cell Bio. 1 (1999) 386-388]. Based on these results, we hypothesize that endogenous lipids which accumulate in SLSD cells due to primary defects in lipid catabolism result in an altered intracellular distribution of cholesterol, and that this alteration in membrane composition then results in defective sorting and transport of SLs. We are currently evaluating potential mechanisms involving SL/cholesterol interactions in the regulation of lipid trafficking in normal and SLSD cells. Experiments using dominant negative constructs of several different Rab proteins are also being carried out to help define the compartment(s) where SL sorting occurs in normal and SLSD cells. Supported by USPHS GrantGM-22942 and the Ara Parseghian Medical Research Foundation.

LIPID SORTING IN THE ENDOCYTIC PATHWAY

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We have examined the endocytic pathways followed by lipids and GPI-anchored proteins following internalization. We have found that lipids enter the same sorting endosomes that contain proteins such as the transferrin receptor or the LDL receptor. As with proteins, lipids can then be delivered to several intracellular destinations including the endocytic recycling compartment (ERC), late endosomes, or the trans Golgi network (TGN). For transmembrane proteins, the best-characterized sorting determinants are cytoplasmic domain peptide sequences. The basis for efficient sorting of lipids remains uncertain, but we have provided evidence that the hydrocarbon tails of lipid analogs are important for sorting of lipid analogs (1, 2). DiI-C16, which prefers more ordered lipid domains, is retained in sorting endosomes as they mature into late endosomes. Little DiI-C16 enters the ERC. In contrast, DiI-C12 and FAST DiI, which prefer less ordered domains are delivered to the ERC after internalization. It is clear, however, that a two-state model for lipid sorting (based on preferences for ordered domains) is much too simple. For example, GPI-anchored proteins, which also prefer more ordered domains, are delivered to the ERC and then exit it slowly for return to the cell surface (3).

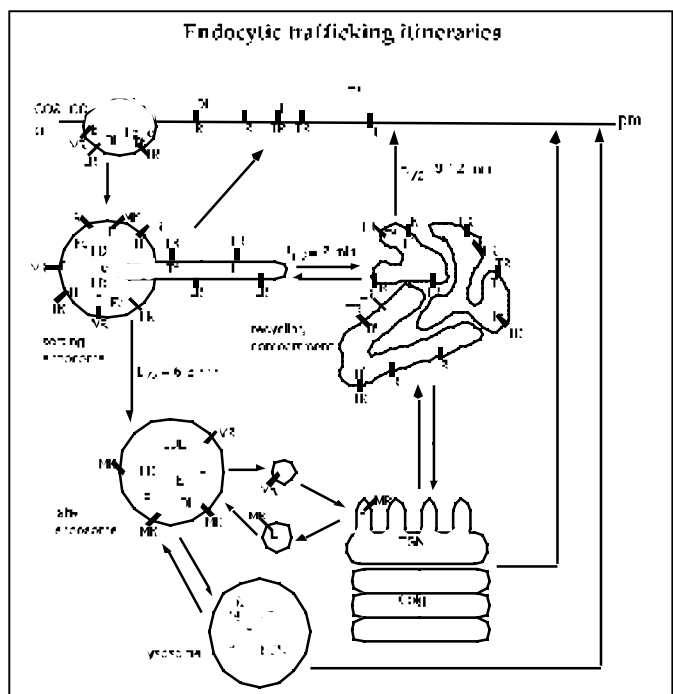
Altering cholesterol levels can change endocytic routing dramatically. When cholesterol levels are lowered about 30% by metabolic depletion, GPI-anchored proteins exit the ERC at the same rate as transferrin receptors (3). Recently, we have found that a similar reduction in cholesterol causes DiI-C16 to be routed to the ERC. Furthermore, this cholesterol depletion causes FAST-DiI and other fluid-domain preferring lipid analogs to form large patches on the plasma membrane. Little FAST-DiI is internalized by cholesterol depleted cells.

Using lipid analogs we have demonstrated that about half of the internalized membrane is recycled to the cell surface with a $t_{1/2}$ of less than 2 minutes (4). This rapid recycling passes through sorting endosomes, but it does not go through the ERC. This amount of rapid recycling implies that membrane internalization is more rapid than previously appreciated, with the half-time for internalization of the entire plasma membrane surface area being as short as 15 minutes.

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Figure 1. *Endocytic trafficking itineraries.* Various receptors concentrate in coated pits which pinch off to form vesicles that fuse into sorting endosomes which are mildly acidic (pH~6.2). Many ligands dissociate from their receptors in the sorting endosome, and the receptors often return rapidly to the cell surface directly or via the endocytic recycling compartment. The released ligands, and most of the solutes that were engulfed, are delivered to late endosomes, which are more acidic (pH~5-5.5) and contain acid hydrolases. Digestion of many proteins occurs in the late endosomes. Over the course of hours, undigested material is delivered to lysosomes. As indicated by the numerous arrows in the diagram, there are multiple documented trafficking pathways among the endocytic organelles, including bi-directional traffic with the Golgi system. Reviewed in (5).



DEFICIENCY IN ETHANALAMINE PLASMALOGEN LEADS TO ALTERED CHOLESTEROL TRANSPORT

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Mammalian cells tightly regulate their cholesterol content and distribution (reviewed in (1)). Cells obtain cholesterol by de novo synthesis and by uptake of plasma lipoproteins. At steady state, most cellular cholesterol resides in the plasma membrane (2). Cholesterol is not static there, but is constantly cycling between the plasma membrane and ER (3). Excess free cellular cholesterol is effluxed to plasma lipoproteins or esterified and stored in lipid droplets. The esterification of excess cholesterol occurs in the ER, and the reaction is catalyzed by the enzyme acyl CoA:cholesterol acyltransferase (ACAT). Thus, the cell's ability to regulate cholesterol homeostasis depends on the transport of cholesterol to the ER.

Studies using inhibitors, disease models, and somatic cell mutants have made progress in defining mechanisms and pathways of intracellular cholesterol movement (1). We have proposed that cholesterol movement from lysosomes to the ER is vesicular based on its inhibition by nigericin, monensin and cytochalasin D (4). The plasma membrane to ER pathway is similarly inhibited by agents that block vesicular transport (5). Transport intermediates have not been identified in either case. Pharmacological evidence suggests that other cholesterol transport pathways proceed independent of vesicular transport pathways. Neither ER to plasma membrane nor lysosome to plasma membrane cholesterol movement are affected by colchicine, cytochalasins, or nigericin (6, 7).

Proteins that modulate intracellular cholesterol transport are currently being identified (1). Specific lipids may also participate in maintaining cholesterol content and distribution, and one example of a lipid participant is sphingomyelin. The plasma membrane has a high sphingomyelin and cholesterol content (2), and there is evidence that the sphingomyelin content helps to maintain the high plasma membrane cholesterol level (8-10). Sphingomyelin digestion causes movement of cholesterol from the plasma membrane to the cell interior (11). Do other lipids also participate in regulating the intracellular distribution of cholesterol?

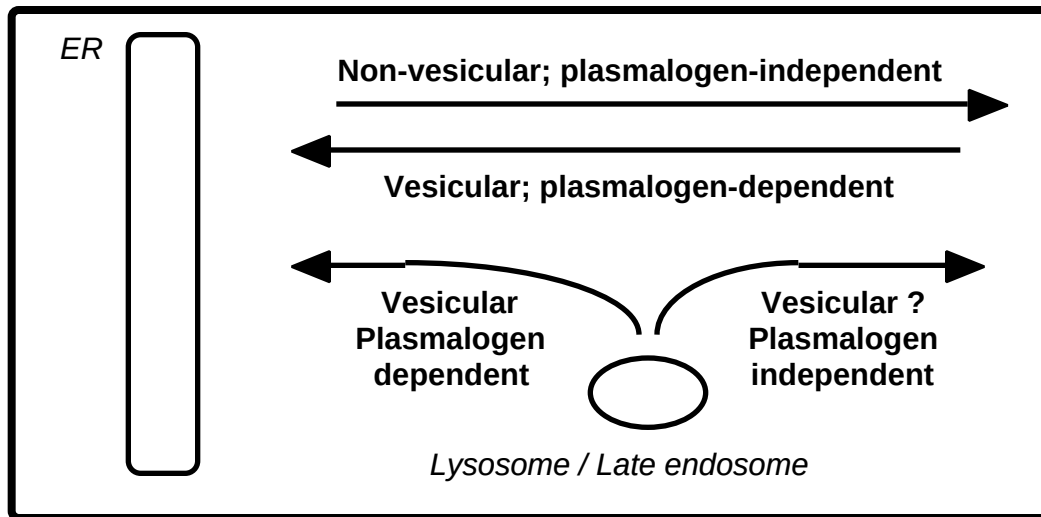
Plasmalogens are a class of phospholipids in which the sn-1 position has a long chain fatty alcohol attached through a vinyl ether bond. Plasmalogens are major ethanolamine and choline phospholipid species, constituting 18% of total phospholipid mass in humans and 11% in Chinese hamster ovary cells (12). Current models suggest that ethanolamine plasmalogens, called plasmenylethanolamine (PlsEtn), play a role in membrane fusion and fission events (13) and protect against reactive oxygen species (14).

We have investigated the role of PlsEtn in cholesterol metabolism. Our study was prompted by the findings that HDL-mediated cholesterol efflux is aberrant in plasmalogen-deficient RAW mutant macrophages (15) and that levels of PlsEtn are reduced in brain tissue from the Niemann-Pick C mouse model (16). Our model systems for this study were CHO cell mutants NRel-4 and NZel-1, which are defective in different steps of plasmalogen biosynthesis (17, 18).

Our analysis revealed that PlsEtn is essential for specific cholesterol transport pathways. The movement of newly synthesized cholesterol from the ER to the plasma membrane was normal in PlsEtn-deficient cells, as was the movement of LDL-derived cholesterol from lysosomes to the plasma membrane. However, the transport of LDL-cholesterol from lysosomes to the ER was deficient, as was the basal movement of plasma membrane cholesterol to ER. LDL stimulation of the ER enzyme ACAT was defective, whereas 25-hydroxycholesterol stimulation of ACAT was normal. Thus cholesterol pathways that are postulated to be vesicular are significantly reduced in PlsEtn-deficient cells. The defective cholesterol transport pathways were restored in NRel-4 cells transfected with a cDNA encoding the missing enzyme, dihydroxyacetonephosphate acyltransferase (DHAPAT).

These data support a role for PlsEtn in cholesterol movement to the cell interior. We do not know if PlsEtn is required at lysosomes or the plasma membrane for the formation of cholesterol-carrying vesicles, or if PlsEtn is required at the ER for fusion of vesicles. The data are consistent with a non-vesicular mechanism of cholesterol transfer from lysosomes to plasma membrane. The evidence for and against vesicular movement of LDL-cholesterol will be discussed, as will the role of the Niemann-Pick type C protein.

What is the mechanism of LDL-cholesterol movement?



Are plasmalogens required for vesicle formation or for cholesterol arrival in the ER?

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BIOGENESIS AND INHERITANCE OF THE GOLGI APPARATUS IN BUDDING YEASTS

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Our goal is to understand how cells make Golgi stacks. According to the cisternal maturation model, new Golgi cisternae form by the fusion of COPII transport vesicles, which are produced at sites of transitional ER (tER). This model suggests that Golgi distribution in budding yeasts is a consequence of tER distribution. To explore this idea, we are studying two closely related yeasts, *Saccharomyces cerevisiae* and *Pichia pastoris*. In *S. cerevisiae*, Golgi cisternae are dispersed throughout the cytoplasm and the entire ER network functions as tER. By contrast, *P. pastoris* has ordered Golgi stacks located next to discrete tER sites. We are analyzing these yeasts by a combination of microscopy and molecular genetics to elucidate the mechanisms that organize the tER-Golgi system.

Because a typical *P. pastoris* cell has only 2 – 5 tER sites, this yeast is an ideal model organism for studying tER dynamics. We labeled the *P. pastoris* tER with a Sec13p-GFP fusion protein and examined living cells by video confocal microscopy. The results indicate that tER sites are relatively stable entities. Remarkably, tER sites do not divide; instead, they form *de novo* and then grow to full size. tER sites diffuse slowly within the ER network, and when two tER sites collide, they fuse. These data suggest that tER sites are composed of molecules that self-assemble into “patches” through weak cooperative interactions.

To explore tER site formation in more detail, we are examining Sec12p, the ER membrane protein that initiates COPII vesicle assembly. Sec12p is found throughout the ER in *S. cerevisiae* but is concentrated at tER sites in *P. pastoris*. By expressing *P. pastoris* Sec12p in *S. cerevisiae* and vice versa, we have shown that (a) *P. pastoris* Sec12p contains a tER localization signal, and (b) this signal is recognized by components that are present in *P. pastoris* but not in *S. cerevisiae*. Ongoing experiments are aimed at characterizing this tER localization signal.

If the tER is the “birthplace” of the Golgi, then Golgi inheritance during cell division may be an indirect consequence of tER inheritance. In *S. cerevisiae*, Golgi elements are present in the daughter cells from early stages of bud formation. A screen for mutants defective in Golgi inheritance yielded multiple alleles of *CDC1*. Our data implicate Cdc1p in various aspects of polarized growth, including the tethering of Golgi elements in the bud. The initial inheritance of Golgi elements may be due to the movement of tER membranes into the bud. Consistent with this idea, the *myo2-66* mutation blocks tER inheritance, and also blocks Golgi inheritance.

Mitotic division of the Golgi apparatus

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During mitosis and cell division it is important that essential cellular components are properly distributed to the two newly-formed daughter cells. The Golgi apparatus undergoes extensive changes during mitosis with stacks of cisternae undergoing vesiculation and ordered dispersal as clusters throughout the cytoplasm. These clusters are segregated to the two daughter cells and are reassembled as a ribbon of stacks around the centrioles at telophase. New insight into these complex morphological changes have been provided by the development of in vitro systems suitable for studying the disassembly and reassembly processes. The rebuilding of stacks requires a Golgi ReAssembly Stacking Protein (GRASP65) which is tightly complexed to another protein, GM130, previously implicated in the docking of transport vesicles. This suggests that stacking can be considered a specialised form of vesicular docking which leads to the formation of stable membrane interactions mediated by the GRASP65/GM130 complex. Both proteins are phosphorylated during mitosis by cyclin B/cdc2 kinase and this could explain the disruption of vesicular docking and cisternal stacking that converts Golgi stacks into Golgi clusters. Two ATPases, NSF and p97, act by two distinct pathways to rebuild Golgi cisternae but they share a common component of the fusion machinery, the t-SNARE, Syntaxin 5. The two pathways are thought to have differing roles in membrane fusion at the rim and core of Golgi cisternae. Sharing a common component would provide a mechanism to allow proper integration of cargo flux through the Golgi apparatus with biogenesis of this organelle.

"TRANSPORT TO THE PLANT VACUOLE:RECEPTORS AND TRANS ELEMENTS"

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Cargo proteins destined for plant vacuoles contain positive sorting signals that lead to their segregation from the default secretory pathway to the cell surface. Two of these sorting signals, an N-terminal propeptide (NTPP) and a C-terminal propeptide (CTPP) can be mechanistically distinguished using the inhibitor wortmanin providing evidence that in some cell types two separate pathways exist for transport to the vacuole. NTPP signals are recognized by the cargo receptor AtELP/BP-80 which causes the cargo to be packaged into clathrin coated vesicles for transport to the prevacuolar compartment (PVC). Transport of protein cargo containing NTPP is accomplished by membrane-bound transport vesicles in a manner similar to trafficking steps throughout the secretory system. Fusion of a transport vesicle with its target organelle requires the presence of a v-SNARE isoform on the vesicle membrane and a t-SNARE isoform found on the target organelle. We have characterized several components of the machinery involved in the sorting of NTPP-type cargo, including a trans-Golgi network (TGN) localized cargo receptor, AtELP, and several SNARE components involved in vesicular traffic between the TGN and the PVC in *Arabidopsis*. We have found that the SNAREs of these organelles are encoded by small gene families, the members of which reside on the same target organelle. Our functional analyses, including gene knock-out data, indicate that the members of these gene families perform distinct essential functions and are not redundant. We believe these distinct functions result from interactions with unique factors that provide specificity. For example, the Sec1p family of proteins are thought to be involved in the regulation of vesicle fusion reactions through interaction with t-SNAREs at the target membrane. AtVPS45 is a member of this family from *Arabidopsis thaliana* that we now demonstrate to be present on the TGN, where it co-localizes with the vacuolar cargo receptor AtELP. Unlike yeast Vps45p, AtVPS45 does not interact with, or co-localize with, the prevacuolar t-SNARE AtPEP12. Instead, AtVPS45 interacts with two t-SNAREs, AtTLG2a and AtTLG2b, that show similarity to the yeast t-SNARE Tlg2p. AtTLG2a and b each co-localize with AtVPS45 at the TGN; however, AtTLG2a is in a different region of the TGN to AtTLG2b by immunogold electron microscopy. We therefore propose that

complexes containing AtVPS45 and either AtTLG2a or b define functional sub-domains of the TGN, and may be required for different trafficking events. Among other Arabidopsis SNAREs, AtVPS45 antibodies preferentially co-precipitate AtVTI1b over the closely-related isoform AtVTI1a, implying that AtVTI1a and AtVTI1b also have distinct functions within the cell. These data point to a functional complexity within the plant secretory pathway, where proteins encoded by gene families have specialized functions, rather than functional redundancy. For this reason, we have started to analyze the interacting factors unique to each gene family member through genomic and proteomic methods.

The C2B-domain of synaptotagmin I is a Ca²⁺-sensing module that controls neuronal exocytosis

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The synaptic vesicle protein synaptotagmin I binds Ca²⁺ and is required for rapid and efficient Ca²⁺-triggered exocytosis. Despite extensive study, the precise function of synaptotagmin in synaptic vesicle trafficking remains the subject of debate and roles in vesicle docking, fusion and recycling have been proposed.

To determine whether and how synaptotagmin functions in the Ca²⁺-triggered fusion of synaptic vesicles we have focused on the Ca²⁺-sensing properties of the protein. Synaptotagmin spans the vesicle membrane once and possesses a large cytoplasmic domain that contains two C2-domains. Multiple Ca²⁺ ions bind to the membrane-proximal C2A-domain. However, it is not known whether the C2B-domain also functions as a Ca²⁺-sensing module, or whether/how Ca²⁺-C2B may function in the release process. Our recent evidence suggests that C2B is a critical Ca²⁺-sensing module that mediates excitation-secretion coupling.

Here we report that the C2B-domain of synaptotagmin binds Ca²⁺ and undergoes conformational changes that drive the homo- and hetero-oligomerization of multiple isoforms of the protein. These effects mirror the divalent cation dependence of neuronal exocytosis. Mutagenesis studies indicate that multiple Ca²⁺ ions bind to a set of redundant binding sites formed by two flexible loops that protrude from one end of C2B. To determine whether Ca²⁺-triggered C2B-mediated oligomerization functions during excitation-secretion coupling, we utilized a dominant-negative approach to inhibit synaptotagmin-clustering in permeable PC12 cells. We observed that a series of recombinant protein fragments, that serve as competitive inhibitors of C2B-mediated clustering, potently inhibited neurotransmitter release during the Ca²⁺-triggered stage of secretion. These data are consistent with a model in which Ca²⁺-triggered synaptotagmin-clustering is a critical step in excitation-secretion coupling.

In a parallel series of studies, we addressed the function of the C2B-domain by characterizing a mutant allele of *Drosophila* synaptotagmin that harbors a single amino acid substitution (tyrosine to an asparagine) within the C2B-domain. We observed that this mutation results in a post-docking defect in exocytosis and have determined the biochemical lesion that gives rise to the mutant phenotype: the mutation selectively inhibits Ca²⁺-driven conformational changes in C2B and virtually abolishes Ca²⁺-driven oligomerization. *Drosophila* harboring this mutant allele also exhibit large decreases in the levels of assembled SNARE-complexes *in vivo*. We further demonstrate that synaptotagmin-oligomers bind SNARE-

complexes and that Ca^{2+} , acting via synaptotagmin, can trigger cross-linking of two SNARE-complexes into dimers. Together, these data indicate that Ca^{2+} -driven oligomerization, via the C2B domain of synaptotagmin, may regulate synaptic vesicle fusion by driving the assembly and clustering of SNARE-complexes. We speculate that these interactions may mediate the abrupt opening and/or subsequent dilation of an exocytotic "fusion pore" in response to Ca^{2+} -transients.

STRUCTURAL INTERMEDIATES IN VIRAL FUSION AND FUSION OF PROTEIN-FREE LIPID BILAYERS

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Specialized fusogenic proteins mediate membrane fusion in viral infection, exocytosis and other biological fusion processes. The best-characterized examples of biological fusion are those used by enveloped viruses, such as influenza virus, to enter their host cells. These fusogenic proteins (e.g., influenza virus hemagglutinin, HA) are identified and well studied. To understand how these proteins induce fusion of membrane lipid bilayers, we dissect the pathway of protein-mediated fusion into distinct stages and study the sequence of conformational changes in the fusogenic proteins upon their activation.

Our working hypothesis (stalk-pore model) is based on earlier work on fusion of protein-free lipid bilayers. We assume that the fusion competence of a lipid bilayer is determined by the propensity of the lipid monolayers to bend. First, the outer (i.e., contacting) monolayers of the membranes bend toward each other to form a stalk - a local hemifusion connection between the membranes. Then a lipidic fusion pore forms and expands in the hemifusion diaphragm formed by the inner monolayers of the membranes. The lipid monolayer's propensity to bend depends on its lipid composition. Thus by adding different lipids to different monolayers of fusing membranes one can probe the net curvature of putative bent fusion intermediates and compare it with that predicted by our working hypothesis. If the stalk-pore hypothesis developed and substantiated for lipid bilayer fusion is applicable to protein-mediated fusion, activated fusogenic proteins should promote bending of the membrane lipid monolayers into stalk and then, pore intermediates. We can make it easier or more difficult for these proteins to do their job by altering the lipid composition of the membranes. Indeed, a number of predictions of the stalk-pore hypothesis were confirmed for HA-mediated fusion and for some other examples of biological fusion.

In our recent work, to study early intermediates of HA-mediated fusion at physiological temperature we slowed down the formation of advanced fusion intermediates by decreasing the number of low pH-activated HA molecules. We identified a new fusion intermediate, which may correspond to a stalk intermediate in our model. While lipid flow between the membranes in this intermediate was restricted, the contacting membrane monolayers were apparently

transiently connected as detected by the transformation of this state into complete fusion with treatments known to destabilize the hemifusion diaphragm. These reversible connections between membranes disappeared within 10 min after low pH application indicating that after the energy released by HA refolding dissipated, the final low pH conformation of HA did not support membrane merger. Since formation of the transient restricted hemifusion sites at physiological temperature was as fast as a fusion pore opening and required less HA, we hypothesize that fusion starts with formation of multiple RH sites, only few of which then evolve to an expanding fusion pore.

Lipid flow between membranes in restricted hemifusion intermediates and in more advanced fusion intermediates such as initial fusion pores is apparently hindered by multiple low pH-activated hemagglutinins. To address the possible role of HA interactions in fusion and HA inactivation we studied the effects of the surface density of HA molecules on the percentage of HA activated at given pH. Using biochemical and functional approaches we found that the rate of low pH activation of Japan and X:31 HA considerably increases with the surface density of HA, suggesting that HA activation is facilitated by either direct or indirect trimer-trimer interactions. Cooperativity of HA activation can facilitate formation of functional multiprotein complexes at a stage of actual fusion.

Our results suggest that HA-mediated fusion, as many other biological fusion reactions, proceeds through the same membrane structures as fusion of protein-free bilayers. Formation of these intermediates, stalks and pores, apparently involves bending of membrane lipid monolayers within a ring-like complex of activated fusion proteins.

IDENTIFYING PROTEINS ESSENTIAL TO Ca^{2+} -TRIGGERED MEMBRANE FUSION

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Despite innovative work identifying numerous proteins and molecular interactions involved in exocytosis, the mechanism of Ca^{2+} -triggered membrane fusion remains unresolved. A major difficulty has been the many interacting membrane trafficking steps in secretion, including membrane retrieval. The challenge has been to analyze a given step in the exocytotic pathway without other steps affecting functional measurements or the identification/isolation of components critical to that step. Defining the specific role(s) of a given protein, beyond its general involvement in exocytosis, has therefore been problematic. Current hypotheses suggest that a SNARE 'core complex' of the proteins VAMP, syntaxin and SNAP-25 constitutes the minimal biological fusogen [1-4]; there is evidence for and against this idea. Definitive identification of essential molecules requires combined biochemical (molecular) and functional (physiological) analyses. A functional, stage-specific physiological membrane preparation would be one direct approach to dissecting the Ca^{2+} -triggered fusion steps of regulated exocytosis. We have been using a minimal system of fully primed, docked and Ca^{2+} -sensitive (fusogenic) secretory vesicles (cortical vesicles, CV) from unfertilized sea urchin eggs, or the isolated CV themselves, to characterize the late events of exocytosis and identify components essential to the fundamental molecular mechanism of Ca^{2+} -triggered membrane fusion. An increase in free Ca^{2+} concentration is thought to cause an interaction between Ca^{2+} ions and specialized Ca^{2+} sensors that leads to activation of the fusion machinery. However, many molecular entities are often lumped together under the terms "fusion machinery", fusion complex, fusogen, and Ca^{2+} sensor. We have established a functional definition of the fusion complex and its activation by Ca^{2+} based on our kinetic analyses. Our working hypothesis is that an increase in the free Ca^{2+} concentration increases the average number of activated fusion complexes, and that these are distributed among CV as a Poisson random variable [5-7]. At sub-optimal Ca^{2+} concentra-

tions, some CV will not have an activated complex and will not fuse; essentially every complex is activated at maximally saturating Ca^{2+} concentrations and all CV fuse. Random removal of fusion complexes should diminish the reserve such that back-extrapolation to the unperturbed level represents the maximum reserve capacity. In terms of concentration and exposure time, both proteases and thiol-reactive reagents irreversibly inhibit fusion with a single exponential decay. At maximally saturating Ca^{2+} concentrations, back-extrapolation indicates a spare capacity of $\sim 7 - 9$ fusion complexes per vesicle at CV-CV and CV-PM docking sites [5,8]. Significant inhibition in the extent of fusion occurs only when reserve capacity is reduced from ~ 9 to < 1 , while an inhibition in rate is predicted for any loss of reserve. After removal of fusion complexes by targeted protein modification, both rate and extent can be used as criteria to identify essential proteins. Preliminary assessments of CV-CV fusion following thiol group modification or protease treatment support this interpretation.

By coupling our Poisson distribution-based mathematical descriptions with biochemical/physiological experiments, we have also been testing whether CV SNARE proteins, or the inter-membrane (*trans*) core complexes, satisfy the criteria of the functionally defined fusion complex. We find that the SNARE complex is not the fusion complex defined for the final, Ca^{2+} -triggered steps of exocytosis: there is no direct correlation between SNARE complex formation or conformational changes and the extent of fusion. Formation of the *trans* complex may modulate the fusion response by promoting a focal inter-membrane contact site, and perhaps by recruiting other critical components [8]. Thus, changes in the SDS-resistant complex are not essential to fusion. However, these studies do not address possible roles of individual SNAREs, other SNARE protein complexes, or other (unknown?) proteins. Further analyses are complicated by the fact that *Clostridial* toxins, which selectively cleave SNAREs, do not completely or consistently block exocytosis. Remaining SNARE fragments may also be functionally relevant. Although most studies show some extent of SNARE cleavage that correlates with a general loss of function, the complete loss of SNAREs has not been quantitatively demonstrated, nor is there evidence of a direct effect on the fusion step of exocytosis. Analysis of the protein sequences for sea urchin ovary VAMP, syntaxin and SNAP-25 indicates a number of trypsin cleavage sites throughout these molecules. Unlike selective SNARE cleavage by *Clostridial* toxins, trypsin destroys these proteins. Importantly, based on sequence analysis, trypsin should also destroy toxin-insensitive SNAREs, and thereby provide a more definitive test for the roles of SNAREs. Trypsin also causes a progressive rightward shift in the Ca^{2+} sensitivity of CV fusion, with a concurrent gradual de-

cline in the extent of fusion that ultimately results in a complete block of the triggered fusion response [8,9]. Thus, sensitivity to trypsin raises the possibility of testing whether the loss of one or more SNAREs correlates with the loss of Ca^{2+} sensitivity and/or fusion. Our preliminary results indicate a correlation of the SNAREs with Ca^{2+} sensitivity [10]. Rather than a direct fusogenic role, we have previously suggested an essential role for the SNAREs in the physiology of exocytosis; complexes may enhance the Ca^{2+} sensitivity of the fusion step, possibly by ensuring a discreet, well-defined site of membrane-membrane contact and thereby ensuring efficient (rapid), triggered fusion at physiological levels of free Ca^{2+} [8,11,12]. Recent electrophysiological studies of chromaffin cells [13] and crayfish neuromuscular junction [14] support these interpretations. Thus, although SNAREs may be necessary for optimal Ca^{2+} sensitivity [8,10], they do not appear to catalyze membrane fusion. A trypsin-sensitive protein other than the SNAREs appears to be essential for Ca^{2+} -triggered membrane fusion. Our efforts to identify proteins essential to Ca^{2+} -triggered fusion is proceeding along lines integrating biochemical, physiological and molecular biological approaches, guided by our continuously developing mathematical description of fusion.

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Molecular basis of Ca²⁺ regulation in neuroendocrine secretory vesicle exocytosis.

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Dramatic progress has been made in elucidating the molecular basis for intracellular membrane fusion. Current models envision the “pinning” of SNARE proteins distributed on vesicle and plasma membrane. The formation of protein complexes in *trans* across the bilayers would promote membrane apposition with subsequent bilayer mixing and fusion. “Priming” reactions on either of the membranes may be required to liberate SNARE proteins to enable *trans* SNARE complex formation, which would occur spontaneously following the completion of “priming”. Constitutive fusion has been recapitulated in vitro by the Rothman lab with proteoliposomes containing complementary sets of SNARE proteins. Neural and endocrine cells, however, contain regulated secretory pathways in which membrane fusion is entirely Ca²⁺-dependent and Ca²⁺-regulated. The basis for Ca²⁺ regulation is currently poorly understood. Biochemical and genetic evidence suggests that synaptotagmins, abundant membrane-spanning, Ca²⁺-binding proteins on secretory vesicles, may mediate Ca²⁺-sensing in regulated membrane fusion.

Using membrane fractions from neuroendocrine PC12 cells that retain competence for Ca²⁺-dependent exocytosis, we found that truncation of the C-terminus of a plasma membrane SNARE protein, SNAP25 (by treatment with the neurotoxin protease, botulinum neurotoxin A), decreased the Ca²⁺-sensitivity of regulated exocytosis. A potential explanation for this was discovered when the C-terminus of SNAP25 was shown to mediate Ca²⁺-dependent binding to synaptotagmin. To determine the relevance of this interaction for Ca²⁺-dependent exocytosis in vivo, SNAP25 mutagenesis studies were conducted. C-terminal SNAP25 acidic residues (D172, D179, E183, D186, D193, E194) were targeted since Ca²⁺-dependent synaptotagmin interactions appear to be ionic and mediated through acidic residues in the interacting protein. These residues in SNAP25 are solvent exposed and unlikely to mediate other essential interactions. Charge reversal mutations were found to progressively inhibit Ca²⁺-dependent synaptotagmin binding to SNAP25 without affecting SNAP25 interactions with syntaxin, VAMP or α SNAP. By developing methods that enable endogenous SNAP25 in PC12 cells to be replaced by specific SNAP25 mutants, we have found that these

mutations confer loss of function for regulated exocytosis. Thus, these studies identify a domain on SNAP25 that is essential for Ca^{2+} -dependent exocytosis and that mediates Ca^{2+} -dependent interactions with synaptotagmin. Overall, the results are consistent with a role for synaptotagmin as a Ca^{2+} sensor for regulated exocytosis in neuroendocrine cells.

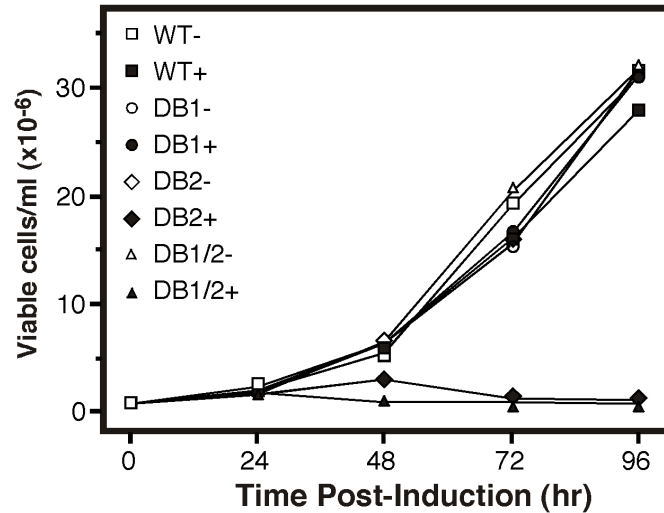
Functional Analysis of a VCP/Cdc48p Homolog in African Trypanosomes with Trans-Dominant Mutants

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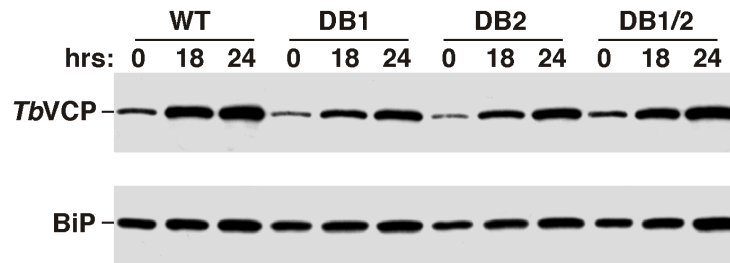
TbVCP is the first member of the AAA (ATPases Associated with a variety of cellular Activities) family of proteins to be identified in African trypanosomes (*Trypanosoma brucei*). Originally identified by degenerate RT-PCR [Roggy, J.L. & J.D. Bangs (1999) *Mol. Biochem. Parasitol.* 98:1-15], *TbVCP* has 72% and 64% amino acid sequence identity with the mouse (VCP) and yeast (Cdc48p) orthologs. Our previous work indicates the trypanosomal ortholog has many of the properties associated with other members of this gene family. *TbVCP* has a 95 kDa monomeric subunit mass; gel filtration and velocity sedimentation analyses are consistent with a native homohexameric structure. *TbVCP* also has *N*-ethylmaleimide (NEM)-sensitive ATPase activity. Finally, cell fractionation indicates *TbVCP* to have a primarily cytosolic distribution with a small membrane-associated pool. VCP and Cdc48p function in membrane fusion events involved in secretory organellar biogenesis and vesicular trafficking; *TbVCP* is presumed to have similar functions in trypanosomes.

Here we report a more detailed genetic analysis of the *TbVCP* locus, and the generation of dominant negative *TbVCP* ATPase mutants for functional studies in transgenic trypanosomes. First, we demonstrate that the *TbVCP* gene complements a yeast *ts cdc48-2* mutant, confirming that *TbVCP* is a functional homolog. As trypanosomes are obligate diploids we next determined that *TbVCP* has a single locus (two copy) genomic organization. One copy, but not both, can be disrupted by targeted homologous recombination suggesting that *TbVCP* is essential for trypanosome viability. Like other *VCP/CDC48* orthologs, the *TbVCP* orf encodes two copies of an ~200 amino acid hallmark AAA ATPase domain, each containing consensus Walker A (binding) and B (hydrolysis) motifs. Site-directed mutagenesis of the DEAD box elements of the Walker B motifs indicates that the second AAA domain is essential for *TbVCP* activity. Constitutive over-expression of *TbVCP* with a single mutation in the second ATP hydrolysis domain (E567Q) or with mutations in both domains (E294Q/E567Q) was not possible, whereas wild type *TbVCP* and the single E294Q mutant had no phenotype. However, regulated over-expression of the E567Q and E294Q/E567Q mutants with a tetracycline-responsive vector was possible resulting in cell death as a trans-dominant phenotype. Cell growth ceased within 24 hours of induction; cells rounded up and death ensued over 4-5 days of culture. Immunofluorescence analyses indicate the onset of progressive fragmentation of the ER at 24 hours consistent with a disruption of organellar biogenesis/maintenance. This work indicates the role of the second ATPase domain in the essential function(s) of this AAA protein. Current efforts are directed at defining the biochemical basis of the trans-dominant phenotype and its effect on secretory trafficking.

A. Effect on Cell Growth



B. Induction of *TbVCP* Protein



Inducible Expression of *TbVCP* Mutants Matched clonal trypanosome cell lines containing the wildtype (WT), E294 (DB1), E567Q (DB2), and E294Q/E567Q (DB1/2) *TbVCP* genes under the control of a Tet-responsive promoter were cultured in the absence (-) or presence (+) of tetracycline (500 ng/ml). **A.** Cell counts were determined at 24 hour intervals. **B.** At the designated times post-induction cells were solubilized in SDS-PAGE sample buffer and analyzed by immunoblotting with anti-*TbVCP* antibody. The blot was then stripped and reprobed with anti-BiP as a control for equal loading. All lanes contain 2.5×10^5 cell equivalents.

**CELL SURFACE DISPLAY AND INTRACELLULAR
TRAFFICKING OF FREE
GLYCOSYLPHOSPHATIDYLINOSITOLS (GPIs) IN
MAMMALIAN CELLS**

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In addition to serving as membrane anchors for cell surface proteins, glycosylphosphatidylinositols (GPIs) can be found abundantly as free glycolipids in mammalian cells (1-3). GPI biosynthesis is a complex pathway involving the sequential addition of monosaccharides to phosphatidylinositol (PI) in the endoplasmic reticulum (ER) (4,5). We have shown that free GPIs are not confined to their site of synthesis, but can redistribute to populate other cellular organelles, including the Golgi and plasma membrane (6). Over short labeling periods (2.5 hr), radiolabeled GPIs were found at similar concentration in all subcellular fractions with the exception of a mitochondria-enriched fraction where GPI concentration was low. Mature triply mannosylated GPIs (H8) were found to accumulate in the plasma membrane after long chase periods and to populate primarily the exoplasmic leaflet of the plasma membrane as detected using periodate oxidation of the cell surface. The lag time for arrival of newly synthesized H8 at the cell surface was slightly slower than the lag time required for secretion of newly synthesized soluble proteins destined for the extracellular space. Transport of GPIs to the cell surface was inhibited by Brefeldin A and blocked at 15°C suggesting that H8 residing in the luminal leaflet of the ER is transported via vesicles through the secretory pathway to the cell surface.

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**Latrotoxin Stimulates Secretion in Permeabilized Chromaffin Cells by
Regulating an Intracellular Ca^{2+} -and ATP-Dependent Event:
a Role for Protein Kinase C**

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α -Latrotoxin, a component of black widow spider venom, stimulates transmitter release from nerve terminals and intact chromaffin cells, and enhances secretion from permeabilized chromaffin cells already maximally stimulated by Ca^{2+} . In this study we demonstrate that chromaffin cells contain a protein antigenically similar to the cloned Ca^{2+} -independent receptor for α -latrotoxin. While this receptor has homology to the secretin family of G-protein-linked receptors, pertussis toxin has no effect on the ability of α -latrotoxin to enhance secretion, suggesting that neither G_i nor G_o is involved in the response. Furthermore, in the absence of Ca^{2+} , α -latrotoxin does not stimulate polyphosphoinositide-specific phospholipase C. α -Latrotoxin specifically enhances ATP-dependent secretion in permeabilized cells. An *in situ* assay for protein kinase C reveals that α -latrotoxin augments the activation of protein kinase C by Ca^{2+} , and use of protein kinase inhibitors demonstrates that this activation is important for the toxin's enhancing effect. This enhancement of secretion requires Ca^{2+} concentrations above 3 μM , and is not supported by Ba^{2+} or non-hydrolyzable guanine nucleotides, which do not stimulate protein kinase C. We conclude that α -latrotoxin stimulates secretion in permeabilized cells by regulating a Ca^{2+} - and ATP-dependent event involving protein kinase C.

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CLONING AND EXPRESSION OF GLYCOLIPID TRANSFER PROTEIN

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Glycolipid transfer protein (GLTP) is a small (23.9 kDa), basic protein (pI $\cong$ 9.0) which accelerates the intermembrane transfer of glycolipids *in vitro*. Recently, we cloned the GLTP cDNA open reading frame (ORF) from bovine and porcine brain using semi-nested RACE-PCR approaches [Lin et al. (2000) *J. Biol. Chem.* 275: 5104-5110]. Heterologous expression in *E. coli* as a GST- or polyhistidine-fusion protein followed by affinity purification yields a fully active protein when assayed for glycolipid intermembrane transfer activity by fluorescence or radio-labeled approaches. The encoded amino acid sequence is nearly identical to the primary sequence previously determined by Edman degradation. Northern analyses show that GLTP mRNA transcripts are uniform in size (~2.2 kb) and widely distributed among bovine tissues [cerebrum > kidney > spleen $\cong$ lung $\cong$ cerebellum > liver > heart muscle]. A similar GLTP transcript distribution was observed by RT-PCR analyses. Cloning of the GLTP cDNA ORF from human skin fibroblasts and from murine epidermal cells indicates a highly conserved, amino acid sequence among mammals without significant homology to other known mammalian proteins. Continuing investigations are assessing the impact of stable GLTP ORF transfection in mammalian cells. Among the issues being addressed are: (1) changes induced in GLTP mRNA transcript levels (Northern analyses); (2) changes induced in protein expression levels (Western analyses); and (3) changes induced in glycosphingolipid composition. [Supported by: NIGMS 45928, Academy of Finland, & The Hormel Foundation]

DYNAMIN-MEDIATED BUDDING OF ENDOCYTIC VESICLES FROM DYNAMIC PLASMA MEMBRANE PLAQUES IN LIVING CELLS

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Dynamin assembles in polymeric rings around the necks of invaginating membrane buds where it is believed to hydrolyze GTP and sever the membrane into discrete vesicles. This model has been formulated from static images which may provide an incomplete view of how dynamin functions in living cells. The **GOAL** of this study was to express dynamin II tagged to green fluorescent protein [Dyn 2(aa)-GFP] in cultured Clone 9 epithelial cells for viewing with time-lapse confocal microscopy. **RESULTS:** Cells expressing Dyn 2(aa)-GFP possessed numerous bright vesicles that were evenly distributed along the dorsal and ventral plasmalemma. In addition, large fluorescent dynamin “plaques” 1-5 microns in diameter were observed along the ventral plasma membrane. Time-lapse imaging of cells revealed that these plaques were extremely dynamic and generated numerous vesicles which were rapidly translocated away into the peripheral cytoplasm. These vesicle-producing plaques appeared to be endocytic structures, which were positive for both clathrin and the endocytic adaptor protein AP2. Electron microscopy of these structures revealed long tubular-vesicular networks which were continuous with the ventral plasma membrane. In cells injected with dynamin antibodies these tubules became constricted into small spherical varicosities, as if prevented from forming discrete individual vesicles. **CONCLUSIONS:** These studies show for the first time that dynamin and other endocytic coat proteins are clustered along the ventral plasma membrane of living cells into plaques which generate large numbers of motile vesicles. Further, dynamin may remain associated with the membrane of a nascent vesicle following its liberation from the cell surface.

EXPECTED AND UNEXPECTED INTRACELLULAR TRAFFICKING OF AGGRECAN DOMAINS. T.-L. L. Chen*, W. Luo+, N. Flay, C. Guo+, M.L. Tanzer+, B.M. Vertel; Dept. Cell Biol. & Anat., FUHS/The Chicago Med. School, No. Chicago, IL 60064; +Dept. BioStruct. & Funct., Univ. Conn. Health Center, Farmington, CT 06030.

Normally aggrecan, the chondroitin sulfate (CS) proteoglycan of cartilage, undergoes numerous synthetic and processing events as it progresses through the exocytotic pathway. Proteins expressed from aggrecan constructs that contain N-terminal sequence through the globular (G)1 domain, a chondroitin sulfate (CS) segment, G3 and an epitope tag follow the typical secretory pathway in transiently transfected CHO cells. Unexpectedly, proteins expressed from a related construct containing the first 23 N-terminal amino acids (the putative aggrecan signal sequence), and lacking G1 (referred to as the G3 construct), primarily accumulate as discrete inclusions in the nucleus, with only 15% of the immunopositive cells typically exhibiting protein in the ER or Golgi. We recently observed that a G3-Green Fluorescent Protein fusion protein also trafficked through the secretory pathway and appeared in the nucleus. When signal-free G3 constructs and those with G3 immediately following the N-terminal signal are expressed, abundant accumulations fill the nucleoplasm in a dispersed pattern. The various nuclear inclusions show differential extractability by detergent. Signal-free G3 proteins accumulate earlier and more extensively in nuclei than those with signals, suggesting nuclear entry from the cytosol via nuclear pores, and further suggesting that the N-terminal signal inefficiently mediates entry of G3 proteins into the ER lumen. Analogous to some neurodegenerative disorders characterized by nuclear inclusions, we observe nuclear colocalization of G3 with Hsp70 and ubiquitin. An association with Hsp25 is an unusual feature of these nuclear inclusions. Immunoelectronmicroscopic characterization also suggests the association of nuclear G3 with other proteins in the inclusions. Collectively, our results raise critical issues about mechanisms regulating intracellular trafficking to the ER and nucleus. (Supported by NIH grants DK28433, AR45909 and the Arthritis Foundation.)

THE FORMATION OF INSOLUBLE OLIGOMERS CORRELATES WITH THE STABLE GOLGI LOCALIZATION OF ST6GAL I

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The ST6Gal I is a sialyltransferase that functions in the late Golgi to modify the N-linked oligosaccharides of glycoproteins. The ST6Gal I is expressed as two isoforms with a single amino acid difference in their catalytic domains (1). The STcys isoform is stably retained in the cell and is predominantly found in the Golgi, while the STtyr isoform is only transiently localized in the Golgi and is cleaved and secreted from a post-Golgi compartment (Fig. 1 and ref 1). These two ST6Gal I isoforms were used to explore the role of the bilayer thickness mechanism and oligomerization in Golgi localization. Analysis of STcys and STtyr proteins with longer transmembrane regions suggested that the bilayer thickness mechanism is not the predominant mechanism used for ST6Gal I Golgi localization (2). In contrast, the formation and quantity of Triton X-100 insoluble oligomers was correlated with the stable or transient localization of the ST6Gal I isoforms in the Golgi (Fig. 2 and ref 2). Nearly 100% of the STcys and only 13% of the STtyr were found as Triton-insoluble oligomers when Golgi membranes of COS-1 cells expressing these proteins were solubilized at pH 6.3, the pH of the late Golgi (Fig. 2, ref 2). In contrast, both proteins were found in the soluble fraction when these membranes were solubilized at pH 8.0. Analysis of other mutants suggested that a conformational change in the catalytic domain, rather than increased disulfide bond-based crosslinking, is the basis for the STcys protein's increased ability to form oligomers and its stable Golgi localization (2).

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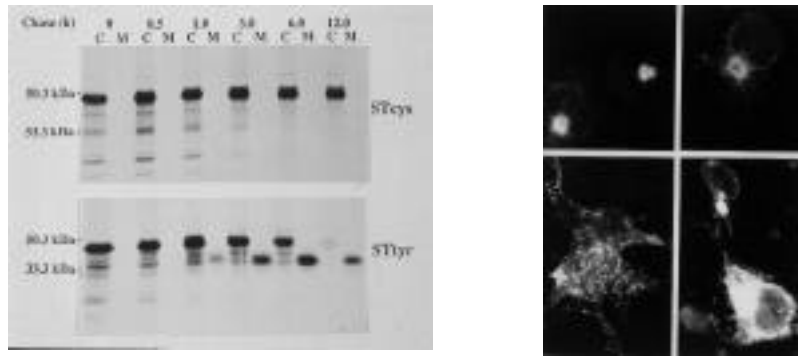


Figure 1. ST6Gal I isoform processing and localization. *Left panel.* COS-1 cells expressing STtyr or STcys were labeled for 30 min with ^{35}S -Protein express labeling mix and chased with media containing unlabeled amino acids for 0-12 h. ST proteins were immunoprecipitated from cell lysates(C) and medium fractions (M), electrophoresed on SDS polyacrylamide gels and visualized by fluorography. *Right panel:* Cells expressing STcys (right panels) or STtyr (left panels) were subjected to indirect immunofluorescence microscopy using an anti-ST6Gal I antibody and goat anti-rabbit IgG-FITC secondary antibody. STtyr is observed in the Golgi of permeabilized cells (top left) and at low levels on the cell surface of unpermeabilized cells (bottom left). STcys is observed in the Golgi of moderately expressing cells (top right) and in the Golgi and ER of highly expressing cells (bottom right). STcys is never observed on the cell surface.

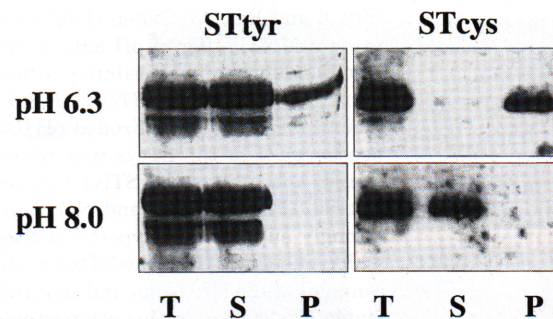


Figure 2. STcys is quantitatively stabilized as insoluble oligomers when Golgi membranes are solubilized at pH 6.3, the pH of the late Golgi. Golgi membranes from COS-1 cells expressing STcys or STtyr were solubilized in 1%Triton X-100 containing buffers of pH 6.3 or 8.0 and separated into soluble (S) and insoluble (P) fractions according to the pH-dependent insolubility assay of Schweitzer *et al.* (*J. Cell Biol.***126**, 25-39 (1994)). One half of the membranes were reserved as total (T) to check recovery. ST proteins were identified by immunoblotting following SDS-PAGE.

ORDERED MEMBRANE INSERTION OF AN ARCHAEAL OPSIN *IN VIVO*
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The prevailing model of polytopic membrane protein insertion is based largely on the *in vitro* analysis of polypeptide chains trapped during insertion by arresting translation. To test this model under conditions of active translation *in vivo*, we have used a kinetic assay to determine the order and timing with which transmembrane segments of bacterioopsin (BO) are inserted into the membrane of the archaeon *Halobacterium salinarum*. BO is the apoprotein of bacteriorhodopsin, a structurally well-characterized protein containing seven transmembrane α -helices (A-G) with an N-out C-in topology. *H. salinarum* strains were constructed that express mutant BO containing a C-terminal His-tag and a single cysteine in one of the four extracellular domains of the protein. Cysteine translocation during BO translation was monitored by pulse-chase radiolabeling and rapid derivatization with a membrane-impermeant, sulfhydryl-specific gel-shift reagent. The results show that the N-terminal domain, the BC loop and the FG loop are translocated in order from the N to the C terminus. Translocation of the DE loop could not be examined because cysteine mutants in this region did not yield a gel shift. The translocation order was confirmed by applying the assay to mutant proteins containing two cysteines in separate extracellular domains. Comparison of the translocation results with *in vivo* measurements of BO elongation indicated that the N-terminal domain and the BC loop are translocated co-translationally, while the FG loop is translocated post-translationally. Together, these results support a sequential, co-translational model of archaeal polytopic membrane protein insertion *in vivo*.

VAC14P REGULATES PRODUCTION OF PHOSPHATIDYLINOSITOL 3,5-BISPHOSPHATE THROUGH ITS INTERACTION WITH THE LIPID KINASE FAB1P

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Membrane fission and fusion are essential for most membrane trafficking events. We have discovered a set of yeast mutants that are defective in vacuolar membrane fission, inheritance, and acidification. The vacuoles of these mutants are large and unlobed, occupying the majority of the cellular volume. These mutants, *vac7*, *vac14*, and *fab1*, have also been shown to be defective in retrograde traffic out of the vacuole (Bryant, et al. 1998 *J. Cell Biol.* 142(3):651-663 and Bonangelino, *et al.*, in preparation). In addition, they contain little to no phosphatidylinositol 3,5-bisphosphate (PI 3,5P₂), a newly identified phospholipid (Gary, et al. 1998 *J. Cell Biol.* 143(1):65-79). Phosphatidylinositol 4,5-bisphosphate is required for membrane trafficking at multiple subcellular locations. PI 3,5P₂ may function similarly in regulating vacuolar membrane trafficking.

Fab1p is the yeast phosphatidylinositol 3-phosphate 5-kinase and is required for the synthesis of PI 3,5P₂. This recently discovered phospholipid is found in low abundance in vegetative cells and increases dramatically in response to osmotic stress. Like *fab1*, mutants *vac7* and *vac14* display low levels of PI 3,5P₂ but the precise roles of the corresponding gene products are unknown. Vac14p is a novel protein with no recognizable motifs. Genetic analysis suggests that Vac14p acts upstream of Fab1p, most likely as an activator during osmotic stress to stimulate the 5-kinase Fab1p to generate more PI 3,5P₂. These studies indicate that changes in vacuolar membrane traffic occur during osmotic stress.

DUAL INTERACTION OF ARF1 WITH SEC7 DOMAIN AND WITH MEMBRANES DURING CATALYSIS OF GDP-GTP EXCHANGE.

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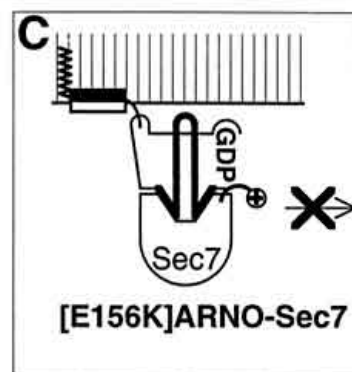
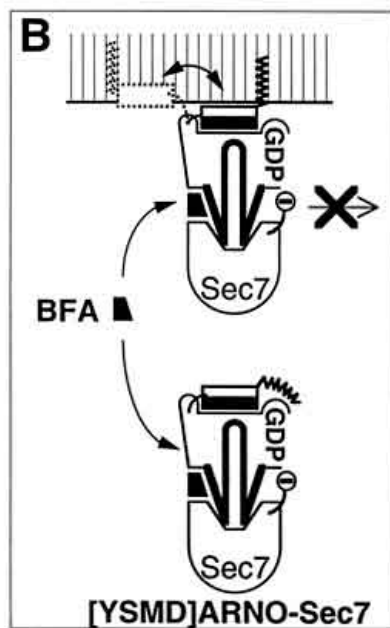
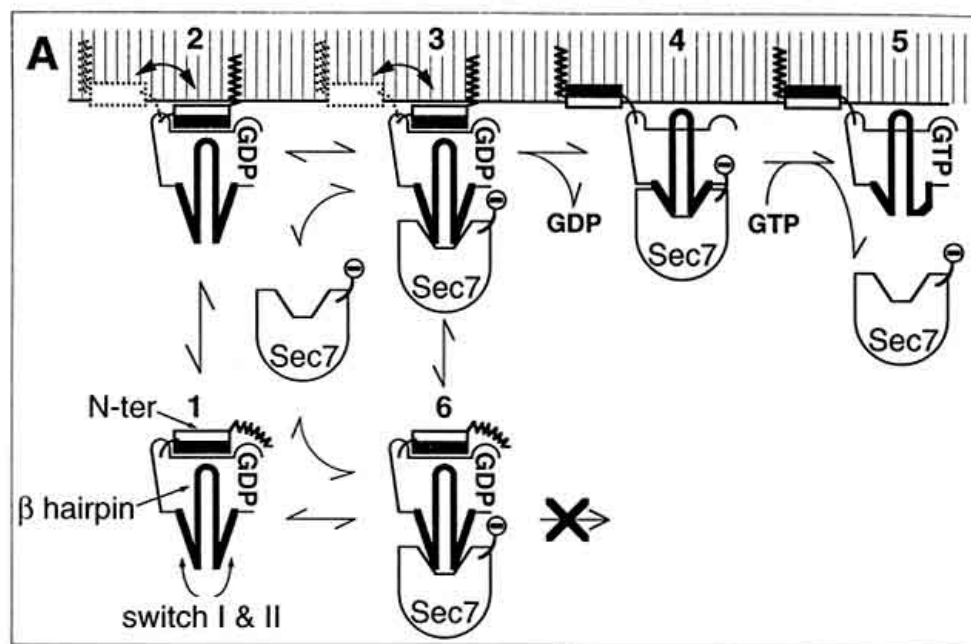
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ARF1 is a small G protein implicated in the maintenance of organelle structure, formation of coated vesicles such as COPI vesicles and clathrine vesicles, and activation of Phospholipase D and PI3K kinases. ARF1-GDP is mostly soluble, whereas ARF1-GTP interacts tightly with membranes. This increase in membrane affinity is due to a change in the exposure of the myristoylated N-terminal helix of ARF1. In the GDP state, the helix is buried in a hydrophobic pocket at the surface of ARF1. In the GTP state the pocket is eliminated and the N-terminal helix interacts with membranes.

GDP/GTP exchange on ARF1 is catalyzed by Sec7 domain proteins. In the ARNO family, the Sec7 domain is flanked by a C-terminal PH domain which interacts with membranes and by an N-terminal coiled-coil domain. Thereby, the GDP-GTP exchange on ARF1 by ARNO proteins occurs on membranes.

Mutagenesis studies and resolution of structures have led to the determination of the mechanism by which Sec7 domains interact with ARF1 and promote the release of GDP. Sec7 domains are characterized by a hydrophobic groove, which binds the switch I and switch II regions of ARF1 and by a "glutamic finger" which promotes GDP release by displacing Mg^{2+} and the β phosphate. The myristoylated N-terminal helix of ARF1 does not participate to the ARF1/Sec7 domain interface.

To determine at which step of the nucleotide exchange reaction the switch of the N-terminal helix of ARF1 occurs, we have studied the binding of functional and abortive complexes between myristoylated ARF1 (myrARF1) and the Sec7 domain of ARNO to liposomes. We show that myrARF1 in complex with ARNO-Sec7, adopts a high affinity state for membranes, similar to the high affinity state of ARF1-GTP. This tight membrane attachment is not only observed when the Sec7d is functional, i.e. promotes the release of GDP, but also when GDP dissociation is blocked because the "glutamic finger" was mutated. In contrast, binding of the uncompetitive inhibitor BFA prevents the ARF1-GDP/Sec7 domain complex from adopting a high affinity state for membranes. These results suggest that the conformational switch of the N-terminal helix of myrARF1 to the membrane bound form is an early event in the nucleotide exchange pathway, and is a prerequisite for a structural rearrangement at the myrARF1/Sec7 domain interface which allows the "glutamic finger" to expel GDP from myrARF1.



A MICROSOMAL GTPase IS INVOLVED IN GLYCOPEPTIDE EXPORT FROM THE MAMMALIAN ER

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Bidirectional transport of proteins via the Sec61p translocon across the endoplasmic reticulum membrane is a recognised component of the ER quality control machinery. Following translocation and engagement by the luminal quality control system, misfolded and unassembled proteins are exported from the ER lumen back to the cytosol for degradation by the proteasome. Additionally, other ER contents, including oligosaccharides, oligopeptides and glycopeptides are efficiently exported from mammalian and yeast systems indicating that bidirectional transport across ER membranes is a general eukaryotic phenomenon¹. Glycopeptide and protein export from ER in *in vitro* systems is both ATP and cytosol-dependent. Using a well established system to study glycopeptide export^{2,3} and conventional liquid chromatography, we isolated a single polypeptide species of 23 kDa from rat liver cytosol that was capable of fully supporting glycopeptide export from rat microsomes in the presence of an ATP-regenerating system (Fig. 1A & B). The protein was identified by mass spectrometric sequence analysis as guanylate kinase (Fig. 1C), a housekeeping enzyme critical in the regulation of cellular GTP levels. We confirmed the ability of GK to substitute for complete cytosol by reconstitution of glycopeptide export from rat liver microsomes using highly purified recombinant GK from *Saccharomyces cerevisiae* (Fig. 2A). Most significantly, we found that GK, and hence cytosolic components, requirement was fully bypassed by low micromolar concentrations of GDP or GTP (Fig. 2B). Similarly, export was inhibited by nonhydrolysable analogues of GDP or GTP indicating a requirement for GTP hydrolysis (Fig. 2B). Membrane integrity was fully maintained under assay conditions as no luminal ER proteins were released. Competence for glycopeptide export was abolished by very mild trypsin treatment of microsomes indicating the presence of an essential protein on the cytosolic face of the ER membrane. These data demonstrate that export of glycopeptide export is controlled by a microsomal GTPase and is independent of cytosolic protein factors.

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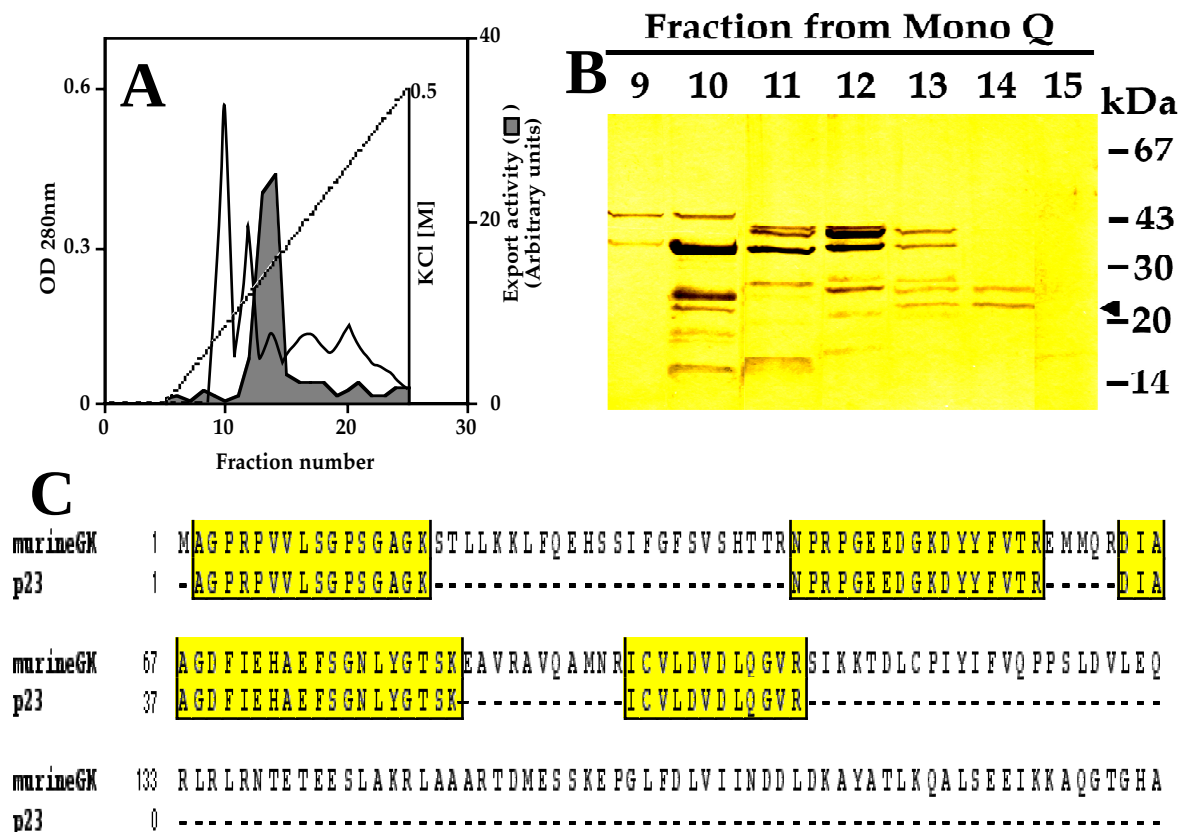


Fig. 1: Purification of the cytosolic glycopeptide exporter from rat liver cytosol. The cytosol from 100,000g supernatant was fractionated by ammonium sulphate precipitation, anion exchange chromatography, gel filtration, hydroxyapatite, hydrophobic intection and finally by anion exchange chromatography on a Mono-Q FPLC column (A). Fractions from Mono-Q was analysed by SDS-PAGE and stained with silver (B). A protein band at ~23kDa (arrowhead) were subjected to in-gel digestion with trypsin and the generated polypeptides were analysed by MALDI and electrospray ionisation mass spectrometry. Peptide sequences were used to identify the protein by searching the NCBI nonredundant database with the programs PROFOUND and revealed homology to mouse guanylate kinase (C).

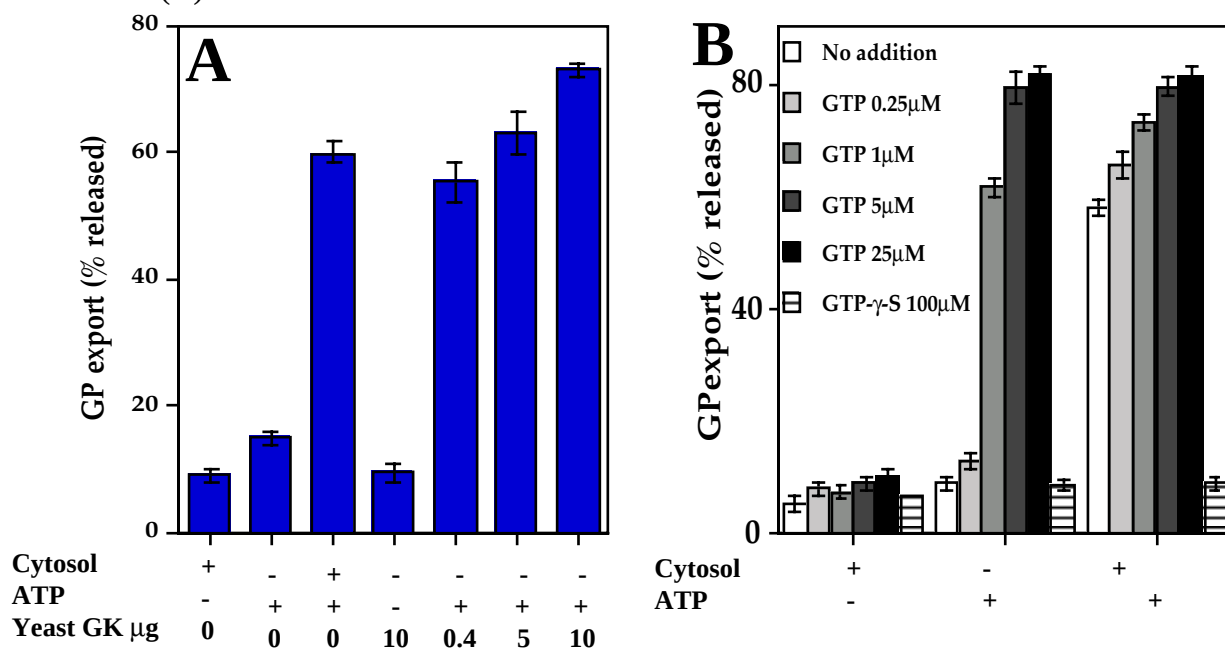


Fig. 2: Cytosol can be bypassed by yeast guanylate kinase (A) or GTP (B). Glycopeptide export from rat liver microsomes (see references 2 and 3 for details) upon incubation at 32 °C for 15 minutes in the presence or absence of reagents as indicated on each graph.

A Novel Mechanism for Localizing Membrane Proteins to the Yeast TGN Requires Function of a Synaptojanin-like Protein

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A-ALP is a model *trans*-Golgi network (TGN) protein in *Saccharomyces cerevisiae*. Its localization to the TGN is mediated in part by a mechanism for its retrieval from a prevacuolar compartment (PVC) that is dependent on a phenylalanine-based signal between positions 81-91 of the cytosolic domain. Structural features outside of the 81-91 region also contribute to TGN localization, but do so through a mechanism distinct from the PVC retrieval step, possibly via static retention. Both the cis-acting signals and trans-acting machinery that mediate the second localization mechanism are poorly understood.

A mutant form of A-ALP, called A(F->A)-ALP, has been shown to be specifically defective for PVC retrieval but normal for the second localization mechanism. A(F->A)-ALP is mislocalized to the vacuole at a moderate rate due to its inability to be retrieved back to the TGN. In order to identify the molecular machinery responsible for PVC-independent localization mechanism, we performed a genetic screen for yeast mutants that exhibited an increased rate of transport of A(F->A)-ALP to the vacuole. One of the mutants obtained in this screen that had a dramatically increased rate of vacuolar delivery of newly synthesized A(F->A)-ALP was *inp53-231*. The *inp53-231* mutant also exhibited defects in retention of another TGN resident protein, Kex2p. However, the *inp53-231* mutant exhibits normal growth and normal intracellular CPY trafficking, suggesting that Inp53p is only required for trafficking of a subset of membrane proteins in the TGN/endosomal system. *INP53* encodes a type II inositol 5-phosphatase that has two distinct domains with different enzymatic activities: a SAC1-domain with a polyphosphoinositide phosphatase activity and an inositol-5-phosphatase domain. *INP53* is a yeast homologue of synaptojanin, an inositol-5-phosphatase that cooperates with dynamin in presynaptic vesicle recycling at the nerve terminal. Therefore, our results support the idea that phosphoinositide metabolism plays an important role in membrane protein trafficking events in the TGN/endosomal system of yeast. Experiments aimed at more precisely characterizing the trafficking step that Inp53p is involved in, and determining the relative roles of the Inp53p domains are currently being carried out.

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SUBUNIT STRUCTURE OF A MAMMALIAN ER/GOLGI SNARE COMPLEX

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SNARE proteins on opposing membranes form complexes that play an essential role in membrane fusion. Although the structure of the SNARE complex involved in exocytosis is known in detail, little is known about the subunit makeup and structure of other SNARE complexes involved in intracellular fusion events. Syntaxin 5 is a mammalian SNARE required for ER-to-Golgi and possibly intra-Golgi transport. In cellular detergent extracts syntaxin 5 is found in complex(es) with near stoichiometric levels of several other ER/Golgi SNAREs, including membrin, sec22b and rbet1. To understand ER and Golgi trafficking it will be essential to know which of these proteins can assemble simultaneously to form a fusion complex. Do these four proteins form a single quaternary complex, or do they form two or more overlapping ternary complexes, each perhaps involved in distinct events? Here we demonstrate that purified recombinant syntaxin 5, membrin, rbet1 and sec22b can assemble to form a single, quaternary complex. Sec22b binding requires all three of the other proteins--it does not participate significantly in ternary or partial complexes. Thus, sec22b appears to associate with the complex last. Perhaps *In vivo*, syntaxin 5, membrin and rbet1 assemble together on one membrane to serve as a high affinity binding site for sec22b on the opposing membrane. The resulting quaternary complex would allow fusion between ER derived vesicles and early VTCs, or alternatively for homotypic fusion of ER-derived vesicles. The ER/Golgi quaternary complex is similar to the synaptic ternary SNARE complex in that it appears to involve four interacting helices; in each case three "Q" helices assemble to create a high affinity binding site for a fourth "R" helix. In the ER/Golgi quaternary complex, one helix each from membrin and rbet1 together appear to play the structural role played by the two SNAP-25 helices in the synaptic complex. SNAP-25 can replace *both* membrin and rbet1 in complexes with syntaxin 5 and sec22b.

INTRACELLULAR TRANSPORT AND CANALICULAR ENRICHMENT OF PHOSPHATIDYLCHOLINE IN POLARIZED HEPG2 CELLS

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Phosphatidylcholine (PC) contributes to about 90% of phospholipids secreted into the bile by hepatocytes. The intrahepatic transport of PC and the mechanism of its release into the bile are largely unknown [1]. Here we have investigated the intracellular transport and canalicular enrichment of fluorescent PC in polarized HepG2 cells. The suitability of HepG2 cells for studying hepatobiliary transport was confirmed by the efficient transport of fluorescent bile salts to the apical vacuole, which resembles the biliary canaliculus (BC) of hepatocytes [2]. Fluorescent analogues of PC became highly enriched in the BC of HepG2 cells and were located on the luminal side of the canalicular membrane as probed for C6-NBD-PC by the dithionite assay. Colabeling cells with the PC analogue β -BODIPY-PC and fluorescent transferrin, which is a marker for the endocytic recycling compartment (ERC) [3], shows that both probes colocalize in vesicles, in a perinuclear compartment (PNC) in non-polarized and a subapical compartment (SAC) in polarized HepG2 cells [4]. This indicates that (i) the PNC and the SAC are components of the ERC and (ii) the analogues of PC enter the ERC in HepG2 cells [5]. Using time lapse video microscopy and digital image analysis evidence for traffic of vesicles containing β -BODIPY-PC between the basolateral domain, the ERC and the BC was obtained. Remarkably, by measuring the kinetics of transport of β -BODIPY-PC from the basolateral plasma membrane, it was found that enrichment of fluorescent PC in the BC ($t_{1/2}$ =3.54 min) precedes its accumulation in the ERC ($t_{1/2}$ =18.5 min). Moreover, no directed vesicle movement was observed during the initial phase of BC labeling, and vesicular transport in the steady state was slower than the kinetics of accumulation of fluorescent PC in the BC. Our results suggest that a vesicular transport via the ERC and a non-vesicular transport pathway to the canalicular membrane exist in hepatocytic cells.

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THE HOG MAP KINASE PATHWAY APPEARS TO ACT
THROUGH ERD1P TO REGULATE THE LOCALIZATION OF A
GOLGI GLYCOSYLTRANSFERASE

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We previously reported the isolation of *ldr* mutants that are defective in the luminal domain retention of the Golgi glycosyltransferase alpha-1,3-mannosyltransferase (Mnn1p). One of which carried a mutation in *HOG1*, which encodes the MAP kinase involved in the high glycerol response pathway in *Saccharomyces cerevisiae* (JCB 1998. 143:935-946). Here we report the identification of *ldr1* as *erd1*. We further report that the expression of Erd1p is not affected in a *hog1* strain. Interestingly, hyperactivation of the HOG1 pathway by overexpression of *PBS2*, encoding the MAP kinase kinase in the HOG pathway, results in the suppression of the mutant secretion phenotype in one *erd1* mutant but not three others. Additionally, this suppression is not observed in the *erd1* null strain. This data suggests that Hog1p is exerting its influence on Mnn1p localization through Erd1p. This work is funded by NIH grant #RO1-50409.

FUNCTIONAL ANALYSIS OF DRS2P --- A POTENTIAL AMINOPHOSPHOLIPID TRANSLOCASE IN YEAST LATE GOLGI

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An *arf1* Δ synthetic lethal screen identified *SWA3/DRS2*, which encodes an integral membrane P-type ATPase and potential aminophospholipid translocase. The *drs2* null allele is also synthetically lethal with clathrin heavy chain (*chc1*) temperature-sensitive alleles, but not with mutations in COPI subunits or other *SEC* genes tested. Consistent with these genetic analyses, we found that the *drs2* Δ mutant exhibits late Golgi defects that may result from a loss of clathrin function at this compartment.

Drs2p is 47% identical to ATPase II from mammalian cells, which is thought to be an ATP-dependent aminophospholipid translocase. However, no direct evidence has been presented yet to indicate that either ATPase II or Drs2p has such function. We are interested in the relationship between the function of Drs2p in the secretory pathway and its potential flippase activity. Towards this goal, late Golgi membranes are purified from wild type and *drs2* Δ cells and assayed for ATPase and aminophospholipid translocase activities. We have found that the specific ATPase activity of *drs2* Δ Golgi membrane is reduced relative to that of WT Golgi membranes. Interestingly, late Golgi membranes contain an ATP-dependent phosphatidylserine (PS) translocase that 'flops' a fluorescent PS derivative (NBD-PS) from the cytoplasmic leaflet of the membrane to the luminal leaflet. This activity is also reduced in the *drs2* Δ Golgi membranes.

PROTONATION OF THE GLOBULAR DOMAIN OF INFLUENZA HEMAGGLUTININ AND ITS RELEVANCE TO THE pH-INDUCED FUSION

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It has been shown that the rearrangement of the HA1 globular domain of influenza hemagglutinin (HA) is one of the initial steps of conformational changes of the HA ectodomain, enabling the formation of the coiled-coil structure of HA2 domain [1-3]. The latter is believed to be responsible for triggering fusion between viral membrane and target membrane [4-7]. Here, a mechanism based on protein protonation is proposed to account for the dissociation of HA1 globular domain and give insight into the pH and temperature dependence of HA-mediated membrane fusion. The preliminary analysis based on molecular modeling shows that the electrostatic interaction between the HA1 and the HA2 domain is the major contribution to the association of HA monomers. Enhancement of protonation of HA1 globular domain at low pH is thought to increase its positive charge, thus, the repulsion between the domain monomers, and this leads to their (partial) dissociation. Based on our proposed mechanism, it is suggested that at the threshold of fusion depending on pH and temperature [8] the electrostatic state of the HA1 globular domain determined by protonation, which is characterized by the number of associated protons on thermodynamic average [9, 10], should be similar. Our results provide solid support for the hypothesis that rearrangement of HA1 domain of the HA trimer is essential for HA-mediated fusion.

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PROTEIN-LIPID INTERACTIONS IN THE ASSEMBLY OF CD1d1 IN VIVO

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CD1d1 belongs to the CD1 group of evolutionarily conserved lipid and glycolipid antigen presenting molecules. It controls the function of natural T lymphocytes whose predicted role is immune regulation. Our biochemical studies revealed that the deep, exclusively hydrophobic groove of mouse CD1d1 contains a cellular glycolipid that was identified as glycosylphosphatidylinositol (GPI). Because membrane-anchored and soluble CD1d1 contained GPI, their association was predicted to occur during the assembly of CD1d1, presumably in the endoplasmic reticulum. Thus the role of cellular glycolipids in CD1d1 structure and function was further elucidated by biochemical means. The data revealed that the two engineered CD1d1 mutants, one defective in recycling from-and-to the plasma membrane and the other defective in efficiently negotiating the secretory pathway, were targeted to the appropriate cellular compartments. The wild type and the two mutant CD1d1 molecules associate with cellular phospholipids and/or glycolipids during biosynthesis. Additional data revealed that wild type CD1d1 expressed by *PigA*-deficient cells unable to synthesise *N*-acetylglucosaminyl-PI, the first glycosylated product in GPI biosynthesis, are thermostable because they assemble with cellular phospholipids and presumably also with cellular glycolipids other than GPI. Moreover, consistent with the complete assembly of thermostable CD1d1, they egress from the endoplasmic reticulum and negotiate the secretory pathway in both *PigA*-positive and *PigA*-deficient cells with similar kinetics. Thus cellular phospholipids and glycolipids play a chaperone-like role in the assembly of CD1d1, presumably by protecting its hydrophobic antigen-binding groove until these lipids are exchanged for a glycolipid antigen(s) within the low pH recycling compartment for display on the plasma membrane.

CHARACTERIZATION OF *ARABIDOPSIS* 68 kDa DYNAMIN-LIKE PROTEIN MUTANTS

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Dynamin and dynamin-related proteins are a family of structurally related but functionally diverse high molecular weight GTP-binding proteins. Many of these proteins have been demonstrated in yeast and mammalian cells to play a role in the formation of endocytic and exocytic transport vesicles. In soybean, a 68 kDa Dynamin-like protein, known as phragmoplastin, is associated with the cell-plate. To determine the precise function of dynamin-like proteins in cell-plate formation we have identified insertional mutants in genes encoding *Arabidopsis* dynamin-like proteins related to phragmoplastin. We have isolated three recessive *Arabidopsis* T-DNA-tagged alleles of *ADL1A*. Mature homozygous *adl1A* embryos have a normal body plan but are much smaller than wild-type embryos and the mutant seeds are shrunken. In mutant *adl1A* embryos the plasma membrane and cell wall between ontogenetically related sister cells is crenulated or wavy indicating a problem in cell-plate formation and/or plasma membrane maintenance perhaps due to a problem in membrane and protein recycling. *adl1A* mutant seeds germinate and appear to undergo normal chloroplast development. However, root and shoot meristematic activity was severely limited. The *adl1A* mutants died at varying times after germination. Development of *adl1A* seedlings could be rescued in tissue culture under conditions that induce the expression of several other *ADL1A*-like genes. Our preliminary characterization of the *adl1A* mutants indicates that ADL1Ap is essential for normal plant development and that there is functional overlap between ADL1Ap and one or more of the other 68 kDa dynamin-like proteins in *Arabidopsis*.

TRANSMEMBRANE PEPTIDES INDUCE FLIP-FLOP OF PHOSPHOLIPIDS IN VESICLES

A model for phospholipid translocation across the *E. coli* inner membrane?

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The enteric bacterium *E. coli* is a fast growing organism. In order to sustain growth, phospholipids synthesized in the cytoplasmic leaflet of the inner membrane have to be translocated to the outer leaflet, and transported to the inner leaflet of the outer membrane. The mechanisms by which these processes occur are unknown.

Phospholipid translocation (or flip-flop) is very slow in model membranes consisting of phospholipids only. In contrast, the rate of flip-flop in isolated *E. coli* inner membranes is high. Therefore the existence of dedicated proteins mediating this process, so-called flippases, has been postulated. However, despite many efforts such a flippase has not been identified yet. Alternatively, the mere presence of membrane proteins without a specific phospholipid transport function might facilitate flip-flop.

To explore the latter possibility, a transmembrane model peptide was incorporated in vesicles consisting of *E. coli* phospholipids. Flip-flop was monitored with fluorescence using various NBD-phospholipid analogues, by assaying their accessibility for dithionite reduction. Various model peptides (differing in length, net charge, etc.) were tested for their ability to induce flip-flop.

Our results indicate that the presence of some of these model peptides in the vesicles facilitates translocation of the NBD-phospholipid analogues. Translocation rates are dependent on the concentration of the peptide, and on the phospholipid probe used. By testing several of the model peptides we try to understand which properties of the peptide are important for phospholipid translocation.

INTERACTIONS BETWEEN DYNAMIN AND THE ACTIN BINDING PROTEIN CORTACTIN MODULATE CELL SHAPE

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The dynamin family of large GTPases has been implicated in the formation of nascent vesicles in both the endocytic and secretory pathways. It is believed that dynamin interacts with a variety of cellular proteins to constrict membranes. The actin cytoskeleton has also been implicated in altering membrane shape and form during cell migration, endocytosis, and secretion and has been postulated to work synergistically with dynamin and coat proteins in several of these important processes. We have observed that the cytoplasmic distribution of dynamin changes dramatically in fibroblasts that have been stimulated to undergo migration with a motagen/hormone. In quiescent cells, Dyn 2 associates predominantly with clathrin-coated vesicles at the plasma membrane and the Golgi apparatus. Upon treatment with platelet-derived growth factor (PDGF) to induce cell migration, dynamin becomes markedly associated with membrane ruffles and lamellapodia. Biochemical and morphological studies using antibodies and GFP-tagged dynamin demonstrate an interaction with cortactin. Cortactin is an actin binding protein which contains a well defined SH3 domain. We show that coractin-SH3 domain can associate with the proline rich domain (PRD) of dynamin. Functional studies support these findings and demonstrate that transfected cells expressing Dyn 2 lacking the PRD (Dyn 2(aa) ΔPRD) do not show wild type distribution. Interestingly, these mutant cells are viable but display dramatic alterations in shape and form. This change in shape appears to be due, in part, to a striking increase in the number of actin stress fibers. These findings provide the first demonstration that dynamin can interact with the actin cytoskeleton to regulate actin reorganization and subsequently cell shape.

RAPID FLIP-FLOP OF PHOSPHOLIPIDS IN ENDOPLASMIC RETICULUM MEMBRANES STUDIED BY A STOPPED-FLOW APPROACH

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The transbilayer movement of short-chain spin-labelled and fluorescent 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) phospholipid analogues in rat liver microsomes is measured by stopped-flow mixing of labelled microsomes with bovine serum albumin (BSA) solution. Extraction of analogues from the outer leaflet of microsomes to BSA can be directly monitored in conjunction with electron paramagnetic resonance (EPR) or fluorescence spectroscopy by taking advantage of the fact that the signal of spin-labelled or fluorescent analogues bound to BSA is different from that of the analogues inserted into membranes. From the signal kinetics, the transbilayer movement as well as the distribution of analogues in microsomal membranes can be derived provided the extraction of analogues by BSA is much faster in comparison to the transbilayer movement of analogues. Half-times of the back-exchange for spin-labelled and fluorescent analogues were < 3.5 s and < 9.5 s, respectively. The unprecedented time resolution of the assay revealed that the transbilayer movement of spin-labelled analogues is much faster than previously reported. The half-time of the movement was about 16 s or even less at room temperature. Transmembrane movement of NBD-labelled analogues was 6- to 8-fold slower than that of spin-labelled analogues.

α -SNAP alters SNARE protein interactions and inhibits a late Ca^{2+} -dependent step of regulated exocytosis

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The plasma membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins syntaxin and synaptosome-associated protein of 25 kDa (SNAP25) and the vesicle-associated membrane protein (VAMP) are essential for a late Ca^{2+} -dependent step in regulated exocytosis. However, their precise roles and regulation by Ca^{2+} remain poorly understood. Previously we have shown that synaptotagmin, a proposed Ca^{2+} sensor for exocytosis, binds to SNAP25, SNAP25-syntaxin binary complexes, and SNAP25-syntaxin-VAMP ternary SNARE complexes in a Ca^{2+} -stimulated manner (Gerona et al., *J. Biol. Chem.* **275**, 6328-6336), interactions that may serve an essential role in this late Ca^{2+} -dependent step in exocytosis. Recently, we have observed that the addition of the α isoform of the soluble N-ethylmaleimide-sensitive factor attachment protein (α -SNAP) inhibits this late step in PC12 cell membranes. To test the hypothesis that this inhibition is the result of disrupting the interaction between synaptotagmin and SNARE proteins at this late step, we compared the ability of an α -SNAP mutant (α -SNAP 41-295), which has been reported to have reduced ability to bind to SNARE proteins, to inhibit regulated exocytosis compared to wild-type α -SNAP. As expected, α -SNAP 41-295 displayed greatly reduced binding to the SNARE proteins SNAP25 and syntaxin, as well as to the ternary SNARE complex, compared to wild-type α -SNAP. The addition of α -SNAP 41-295 to the late Ca^{2+} -dependent step of exocytosis in PC12 cell membranes did not result in the inhibition seen by the addition of wild-type α -SNAP. These results suggest that α -SNAP inhibition of the late Ca^{2+} -dependent step of regulated exocytosis results from the ability of α -SNAP to inhibit the interaction of the SNARE proteins with other proteins, including the proposed Ca^{2+} sensor synaptotagmin.

CHARACTERIZATION OF THE GOLGI TETHERING FACTOR SEC34P.

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Vesicular transport between membrane compartments requires high specificity and tight regulation to deliver cargo molecules to the correct acceptor organelle and maintain the integrity of distinct compartments. To ensure the proper directionality of membrane flow, the target organelle must possess appropriate molecular machinery allowing for specific recognition and docking of the incoming vesicle. Vesicle fusion with specific compartments is mediated by interaction between integral membrane proteins on vesicular and target membranes, termed v-SNAREs and t-SNAREs, respectively. Interaction of v- and t-SNAREs is promiscuous and could not account by itself for high fidelity of intracellular trafficking. The ‘tethering’ factors and small GTPases of Rab family are thought to ensure the specificity of vesicle targeting.

Sec34p, a peripheral membrane protein, is one of the putative ‘tethering’ factors that acts at the ER to Golgi transport step of the secretory pathway. Sec34p forms a complex with another ‘tethering’ factor Sec35p (VanRheenen et al., 1998, 1999). Sec34p is identical to GRD20 – a factor that is required for protein sorting in the trans-Golgi network/endosomal system and for polarization of the actin cytoskeleton (Spelbrink and Nothwehr, 1999). Interestingly, SEC34/GRD20 mutants tend to mislocalize Golgi proteins like the v-SNARE VTI1 that is involved in retrograde trafficking and interacts with the cis-Golgi t-SNARE Sed5p (Lupashin et al., 1997). We have discovered that Sec34p specifically physically interacts with the cis Golgi SNARE Sed5p both *in vitro* and *in vivo* and may regulate the activity of that SNARE for both anterograde and retrograde trafficking. Sec34p can be co-precipitated with distinct subset of the cis-Golgi v-SNARE proteins including Ykt6p and Sec22p, but not with Bet1p and Bos1p, suggesting that Sec34/Sec35 complex is primary involved in targeting of the sub-population of cis-Golgi oriented transport vesicles.

Since many secretory factors are evolutionarily conserved, we have explored whether the components of the Sec34p/Sec35p complex were conserved in higher eukaryotes. Several human ESTs were discovered with a high degree of similarity to Sec34p. Based on the primary sequence of the EST z21242 we have successfully amplified a 5’ 900 bp long fragment by using a cDNA library from human placenta. The resulting DNA fragment encoded a 269aa polypeptide highly homologous to Sec34p. A complete human cDNA was obtained by PCR screening of a Rapid-Screen Arrayed Human Fetal Brain cDNA library (OriGene Technologies, Inc.) with hSec34 specific primers. Completely sequenced hSec34 cDNA was 4.5 kb long and contained a 2484 bp ORF that encode 828aa polypeptide homologous to Sec34p.

To study intracellular localization of hSec34p we have constructed GFP-hSec34p hybrid expression vector. An initial observation indicates that GFP-hSec34 protein is localized in the perinuclear region of transiently transfected Hela cells and at least partially co-localized with the cis-Golgi marker p115.

**ENDOPLASMIC RETICULUM MEMBRANE SORTING PROTEIN
BAP31 IS HIGHLY EXPRESSED IN NEURONS AND CERTAIN
ENDOCRINE AND EXOCRINE CELLS.**

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BAP31 was identified in B lymphocytes as a transmembrane protein accompanying nascent membrane immunoglobulin D in early sorting from endoplasmic reticulum to Golgi compartments. A similar role was reported for BAP31 in sorting a v-SNARE protein between the endoplasmic reticulum and the intermediate compartment/cis-Golgi of cultured renal cells. Consistent with a role in early sorting of membrane proteins, BAP31 has a C-terminal endoplasmic reticulum-retrieval dilysine (KKEE) motif. Its RNA is ubiquitous, but the anatomical localization of BAP31 protein has not been determined. To gain further insight into its function we used monoclonal antibodies to localize BAP31 in tissues from human and lower primates. BAP31 immunoreactivity was prominent in cerebellar Purkinje neuron bodies and dendrites, gonadotropic cells of the anterior pituitary, immature but not mature T-lymphocytes, B-lymphocyte-enriched areas of spleen and tonsil, ovarian thecal and follicular cells, proximal but not distal renal tubules and active but not quiescent thyroid epithelium. Adrenal cortex was more immunoreactive than medulla. Blood vessels and skeletal muscle were non-reactive. The anatomical distribution of BAP31 suggests that it may be involved in the early sorting of membrane proteins destined for assembly in macromolecular complexes en route to selected sites of exocytotic and signaling activities.

COOPERATIVE ACTIVATION OF INFLUENZA VIRUS HEMAGGLUTININ DURING VIRAL ENTRY

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Influenza virus entry into the target cell requires major structural rearrangement of the fusion protein, hemagglutinin (HA), which drives fusion of the viral envelope with the endosomal membrane. Such major structural refolding is initiated by low pH-triggered release of the fusion peptide from hydrophobic groove and proceeds to a fusion-competent conformation through a number of poorly understood intermediate structures. While the actual fusion event apparently involves concerted action of multiple HA trimers, early activation has been thought to develop at the level of individual trimers. In the present work we studied the effects of the surface density of HA molecules on the percentage of low pH activated HA. Conformational change in HA from initial to an activated form, was assayed by susceptibility to protease (e.g., thermolysin) digestion and disulfide reduction. The rate of HA activation was also tested by functional assays using fluorescence microscopy. HA-expressing cells were treated with a first low pH pulse in the absence of the target membrane. Such pretreatment resulted in activation and then inactivation of HA molecules. The extent of this activation/inactivation under different conditions was evaluated by subsequent addition of human erythrocytes followed by the second low pH pulse. Generally, the greater HA activation during the first pulse, the fewer HA was available for fusion and thus the lower the extent of fusion. Using biochemical and functional approach we found that HA activation of two different strains of influenza virus, i.e. Japan and X:31, considerably increases with the increase in HA surface density. Such finding supports the notion that HA activation is a cooperative event. This cooperativity can reflect either direct trimer-trimer interaction (e.g., via fusion peptides of adjacent trimers) or trimer interactions with the membrane they are anchored to.

REGULATION OF GPI BIOSYNTHESIS IN *LEISHMANIA* PARASITES

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The cell surface of the protozoan parasite, *Leishmania mexicana* is coated by a number of GPI-anchored macromolecules (glycoproteins, proteophosphoglycans and a lipophosphoglycan (LPG)) and a family of structurally distinct free GPIs. While the LPG is an essential virulence factor, the free GPIs appear to be essential for normal parasite growth. The biosynthesis of the protein and LPG anchors as well as the free GPIs is complex and probably involves three distinct biosynthetic pathways that are developmentally regulated. In order to understand how these pathways are regulated we have investigated whether one or more of these pathways are compartmentalized within the ER. By subcellular fractionation, we showed that all three GPIs are synthesized in a light microsomal compartment which is distinct from the bulk ER membrane. To further characterize this compartment we have tagged a number of GPI enzymes with GFP and expressed these chimeras in *L. mexicana* promastigotes. Unexpectedly, we found that only a small pool of these chimeras are retained with the ER, while a major pool is targeted to a novel tubular organelle that extends from the anterior end of the cell (the sole site of exo- and endocytosis) to the posterior end of the cell. We show that this tubular organelle is a mature lysosomal compartment based on the finding; 1) that it has a multivesicular structure and is connected to the *trans* face of the Golgi apparatus via a series of uniform multivesicular bodies, 2) GFP chimeras that reach this compartment are degraded to a protease-resistant GFP core, 2) these structures are perturbed by several lysomorphotropic agents and 4) vital lipid stains for endocytic membrane transport reach the tubule after first labeling a network of early endosomes. These studies define a new lysosomal compartment in *Leishmania* and suggest that protein transport from the ER to this multivesicular tubule (MVT) plays an important role in regulating the cellular levels of ER glycosylation pathways.

INTERACTIONS WITHIN THE YEAST t-SNARE Sso1p THAT CONTROL SNARE COMPLEX ASSEMBLY

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Growth of *Saccharomyces cerevisiae* cells is polarized, occurring exclusively in the newly formed and developing bud. Yeast cells accomplish this by restricting exocytosis to bud tips and, at a stage just before cytokinesis, to mother-bud necks. Surprisingly, the relevant t-SNAREs (Sso and Sec9p) are not localized exclusively to sites of exocytosis, but are broadly distributed over the inner surface of the plasma membrane. In addition, only low levels of binary complex are present at steady state *in vivo*. These observations raise the possibility that assembly of binary, and hence ternary, SNARE complexes is regulated. We have previously shown that the rate of binary SNARE complex assembly is extremely slow *in vitro*; therefore, it is logical to propose that this process is under kinetic control *in vivo* and that it is accelerated by proteins localized to sites of secretion.

In vitro, Sso1p exists in a “closed” conformation that inhibits its entry into complexes with Sec9p. In this closed conformation, the N-terminal inhibitory domain (NT) binds the C-terminal SNARE motif (CT). *In vivo*, regulatory proteins presumably cause a conformational change in Sso, whereby CT is released from NT, thus allowing binary complex formation. We have tested this proposed regulatory mechanism by showing that the intramolecular interaction between the NT and CT is sensitive to perturbation, and that destabilizing this interaction accelerates SNARE assembly. Using alanine-scanning mutagenesis combined with kinetic assays of binary complex formation, we have identified a conserved hydrophobic groove on the regulatory NT domain that interacts with CT residues in the closed conformation. We have also shown that yeast lacking the regulatory domain of Sso1p are inviable, suggesting that unregulated SNARE assembly may be deleterious *in vivo*.

DIRECT INTERACTION BETWEEN CAVEOLIN AND DYNAMIN

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Caveolae are flask-shaped invaginations of the plasma membrane found in many different cell types. Functional studies demonstrate that caveolae play an important role in the potentiation of cellular signaling cascades. Caveolins (Cav-1, Cav-2), are the major structural components of caveolae, and are believed to bind to each other and to intracellular signal transducers, e.g. G-protein-coupled receptors, heterotrimeric G-proteins, receptor tyrosine kinases and eNOS. Caveolae, once thought to be static structures, are now known to be internalized from the plasma membrane. Most recently, we (Henley *et. al.*, JCB, 141(1): 85-99, 1998) and others (Oh *et. al.*, JCB, 141(1) 101-114, 1998) have demonstrated that the scission of caveolae from the plasma membrane is mediated by the large GTPase dynamin. Immunofluorescence and immunoelectron microscopic studies show an intimate association between dynamin and caveolae. How dynamin interacts with caveolae is currently unclear. Therefore, the GOAL of this study is to determine if dynamin and caveolin directly interact and if so which domains in these two proteins facilitate the interaction.

CELL-PLATE ASSEMBLY IN PLANTS: A ROLE FOR CDC48?

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Cell-plate assembly is required during plant cytokinesis to separate the cytosol and organelles into two daughter cells. Formation of the cell-plate involves consolidation and fusion of secretory vesicles across the equatorial center of the cell with expansion and eventual fusion with the parental plasma membrane. Little is known about the molecular participants and fusion events that underlie this process.

An *Arabidopsis thaliana* homolog of the membrane fusion factor Cdc48p/p97 (AtCdc48p) has been identified and cloned (Feiler et al. (1995) EMBO J. 14, 5626). We have generated AtCdc48p-specific antibodies to test the hypothesis that this protein may play a role in cell-plate formation. Using standard and confocal immunofluorescence microscopy we have demonstrated that AtCdc48p is associated with small cytoplasmic vesicles and is localized at the cell-plate in a dividing *Arabidopsis* T87 suspension-cultured cells. Biochemical experiments indicate that AtCdc48p is a peripheral membrane protein. The soluble form is present predominantly in large complexes ranging in size from 17.7-19.4S. AtCdc48p membrane association is sensitive to reducing agents (DTT) and is stabilized by salt. Subcellular fractionation of T87 cells by sucrose gradient sedimentation suggests that membrane-bound AtCdc48p is associated with a slowly sedimenting membrane fraction which also contains the previously identified plant syntaxin homologs AtPep12p, AtSed5p and the putative cell-plate syntaxin, Knolle. *In vitro* binding studies indicate that soluble AtCdc48p from T87 cells is competent to bind *E. coli* produced GST-AtSed5AΔTM only under conditions that require ATP and support its hydrolysis. Binding of AtCdc48p to GST, GST-AtPep12ΔTM and GST-KnolleΔTM was not detected under the experimental conditions used.

We are presently testing (1) whether AtSed5p is localized to the cell-plate, (2) what accessory factors are associated with AtCdc48p to determine its functional specificity and (3) whether there are other “cell-plate” specific syntaxin homologs that interact with AtCdc48p.

YEAST GGA PROTEINS INTERACT WITH GTP-BOUND ARF AND FACILITATE LATE GOLGI TRANSPORT.

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ARF proteins are thought to regulate the formation of transport vesicles at many steps of the secretory and endocytic pathways. A recently identified family of ARF effectors named GGAs appears to regulate membrane traffic exiting the trans-Golgi network in mammalian cells (Boman et al., 2000). We have identified two GGA homologues in the yeast *S. cerevisiae*. These previously uncharacterized open reading frames, YDR358w and YHR108w, were named *GGA1* and *GGA2*, respectively. Using the two-hybrid assay and GST-affinity chromatography, we show that Gga1p and Gga2p interact with yArf1 and yArf2 in a GTP-dependent manner, suggesting that both are functional homologues of the human GGA proteins. We have mapped the Arf-binding domain of Gga1p to a region in the amino-terminal half of Gga1p (amino acids 170-330). The interaction with Arf is independent of the carboxyl-terminal 100 amino acids, which resemble the γ -adaptin ear domain. Gene deletion experiments indicate that *GGA1* and *GGA2* are not essential genes, as single and double knockouts are viable at both 30°C and 37°C. However, cells lacking *GGA1* and *GGA2* exhibit defects in invertase processing and CPY sorting, but not endocytosis. We are now testing whether Gga proteins facilitate anterograde traffic from the late Golgi or aid in retrieval from a post-Golgi compartment.

SYNTAXIN 7 AND IT'S INTERACTING PROTEINS MEDIATE CONTROL OF LATE ENDOSOMAL / LYSOSOMAL FUSION

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In yeast, several proteins have been identified that control vesicular fusion with the vacuole (lysosome), such as SNARE (Soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) and SNARE-interacting proteins. In an effort to understand analogous events regulating mammalian lysosomal biogenesis, mammalian homologues of proteins responsible for vacuole fusion were identified. A functional analysis of these candidate proteins during endosomal / lysosomal fusion was then undertaken.

Syntaxin 7 represents a recently identified member of the t-SNARE family of transport receptors. Using a verity of immunofluorescence and immuno-EM techniques Syntaxin 7 was found to be most abundant in the lysosomal and late endosomal membranes of MDCK cells. Further, to examine the involvement of Syntaxin 7 in late endocytic trafficking, an event previously shown to involve NSF, cytosolic factors and energy, a late endosome to lysosome fusion assay was used. It was found that antibodies specific to Syntaxin 7 inhibited late endosome to lysosome fusion, substantiating the funtional role of Syntaxin 7 in lysosomal biogenesis. Co-immunoprecipitation experiments showed an association of Syntaxin 7 with the endosomal v-SNARE Vamp 8 and immunofluorescence experiments showed that Vamp 8 partially localizes to late endosomes.

In order to understand the mechanisms that regulate the delivery of material to the lysosome, the further characterization of proteins that interact with Syntaxin 7 was also undertaken. One candidate is the mammalian homologue of the yeast Vps11 protein that is part of a multi-subunit complex that is predicted to control SNARE mediated fusion events with the yeast vacuole. We have now cloned the mouse Vps11p homologue and used a recombinant protein to raise specific antibodies. Preliminary data indicates a role for this protein in the regulation of lysosomal biogenesis.

LOCALIZATION OF YEAST AND MAMMALIAN GGA PROTEINS TO THE LATE GOLGI.

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Activated ARF proteins are localized to the cytosolic face of many cellular membranes, including the Golgi, trans-Golgi network (TGN), endosomes, and plasma membrane. A family of ARF effectors in mammalian cells, named GGA proteins, localize to a subset of these membranes, predominantly the TGN. A protein fragment of GGA1 that encompasses the Arf-binding domain retains this localization. Other protein or lipid interactions that mediate the localization of Ggas to the TGN are currently unknown. We have raised antibodies against the two yeast homologues of the GGA proteins named Gga1p and Gga2p. These antibodies recognize their antigen on immunoblots and in immunofluorescence experiments. In yeast, Gga1p and Gga2p each localize to defined puncta that resemble Golgi cisternae. We are currently testing for colocalization with several organelle markers, including the late Golgi protein Kex2p, to identify these puncta. Determinants of the Arf-dependent localization in both yeast and mammalian cells are being investigated.

TOPOLOGY OF GLYCOSYLPHOSPHATIDYLINOSITOL ANCHOR ASSEMBLY IN SUBCELLULAR MEMBRANES OF *TOXOPLASMA GONDII*

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Glycosylphosphatidylinositol (GPI) glycolipids serve as membrane anchor for a large number of cell surface proteins in various eucaryotic cells. These glycolipids are assembled by sequential addition of monosaccharides and phosphoethanolamine to phosphatidylinositol. GPI anchor precursors are transferred to newly synthesized proteins *en bloc* in the lumen of the endoplasmic reticulum (ER). *Toxoplasma gondii* GPI anchors possess a glucose-containing side chain, which has been shown to be highly immunogenic in humans [1]. We are specially interested in the topology of side chain modification before addition to protein. We have established a system of semi-permeabilized *T. gondii* cells to investigate the transmembrane distribution of GPI biosynthetic intermediates. Permeabilized parasites were tested for membrane integrity of organelles (ER, rhoptries). In initial experiments GPI biosynthetic intermediates were labeled with UDP-[³H]GlcNAc in permeabilized parasites, and the transmembrane distribution of the labeled lipids was probed with phosphatidylinositol-specific phospholipase C (PI-PLC). Intermediates with an acyl modification at the inositol, not detectable in *in vivo* labeled preparations, serve as an internal control. Treatment of the radiolabeled parasites with PI-PLC revealed that ~ 70 % of a glycolipid corresponding to glucosaminyl phosphatidylinositol (GlcN-PI) could be hydrolyzed. These data indicate that this lipid is primarily present in the cytoplasmic leaflet of the ER. This is in good agreement with investigations on mammalian cells [2]. Future experiments using the established system of permeabilized *T. gondii* parasites should provide details about the transmembrane distribution of higher glycosylated and modified GPI biosynthetic intermediates.

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ERGOSTEROL TRANSPORT FROM ITS SITE OF SYNTHESIS TO THE
PLASMA MEMBRANE IN *SACCHAROMYCES CEREVISIAE*.

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Sterols are essential elements of eukaryotic plasma membranes involved in membrane and lipoprotein biogenesis, as well as cell growth and membrane domain formation. In mammalian cells, plasma membrane (PM) cholesterol is acquired in two distinct ways: receptor-mediated endocytosis of LDL and transport of *de novo* synthesized cholesterol from the endoplasmic reticulum (ER) to the PM. Information about intracellular cholesterol transport has been largely confined to studies on LDL derived cholesterol where a clear picture has begun to emerge (1). On the other hand, the mechanisms of cholesterol transport from its site of synthesis (ER +/- internal membranes) to the PM remain quite obscure although two hypotheses have been proposed based on limited available data. The first, involves an as yet uncharacterized vesicular transport pathway that bypasses the Golgi apparatus (2) whereas a second hypothesis, based on recent results, suggests that a complex of cytosolic proteins acts as a sterol carrier (3). Our aim is to evaluate these hypotheses using yeast as a model system.

The main sterol in yeast, ergosterol, is a molecule structurally related to cholesterol. Like cholesterol in mammalian cells, ergosterol is primarily localized to the PM in yeast. Ergosterol is synthesized by reactions occurring in the ER with a possible role of lipid particles (4) and, like cholesterol, can be esterified. Yeast represents an excellent model system in which to study the transport of *de novo* synthesized sterols to the PM because of its amenability to biochemical and genetic analyses and because ergosterol is not readily taken up by cells growing under aerobic conditions. As an initial step in the characterization of sterol trafficking and based on spheroplast formation followed by two successive Percoll gradients, we prepared a highly enriched PM fraction that is almost completely devoid of other cellular membranes. We have also isolated and characterized sterols from cells metabolically labeled with [³H]methionine (HPLC, TLC, mass spectroscopy and UV spectrum). Currently we are using the PM isolation procedure, coupled with pulse

metabolic labeling to assess the arrival of metabolically labeled ergosterol at the plasma membrane. These experiments, as well as investigations of ergosterol transport in yeast secretory (*sec*) mutants will be presented.

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SELECTIVE EXPORT OF MHC CLASS I MOLECULES FROM THE ER

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Major Histocompatibility Complex (MHC) class I molecules present peptides to effector T-lymphocytes. MHC class I molecules consist of a transmembrane polymorphic heavy chain, β 2-microglobulin and a 8-10 residue peptide. Loading of MHC class I molecules with peptides occurs in the ER during their association with the transporter associated with antigen processing (TAP). It is assumed that upon peptide loading and dissociation from TAP, MHC class I molecules exit the ER by non selective bulk flow. If this were true, we would predict that rates of ER-to-Golgi transport are limited by dissociation from TAP. We show that MHC class I molecules, H2K^b and H2L^d maintain allele-specific plateaus in their ER-to-Golgi transport, in spite extensive dissociation from TAP upon loading with high affinity peptides. Consistent with a selective model of ER export, peptide-loaded MHC class I molecules concentrate at ER-exit sites, while TAP molecules themselves are excluded from these sites. Allele specific rates of ER-to-Golgi transport appear to be independent of the cytoplasmic tails of MHC class I molecules, which lack ER export motifs. These data suggest that the rate limiting step in the export of MHC class I molecules from the ER is recruitment into COPII vesicles by a mechanism that most likely involves selective association with cargo receptors.

REGULATION OF THE ACTIN CYTOSKELETON DURING VESICLE FORMATION ON THE GOLGI

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We find that the small GTPase, ARF, promotes the assembly of at least two distinct pools of ARF on Golgi membranes (Fucini et al., 2000, *J. Biol. Chem.*, in press). These pools have distinct sensitivity to the actin-depolymerizing toxins cytochalasin D and to latrunculin A. One pool of actin is found to cofractionate with Golgi-derived COPI-coated vesicles and contains the actin-binding proteins, drebrin and spectrin. Disrupting the actin cytoskeleton affects the release of COPI-coated vesicles in a cell-free Golgi-budding assay. We show that actin assembly on Golgi membranes can be reconstituted with recombinant myristoylated ARF1 using a cell-free system. Golgi-membrane-bound recombinant ARF1 is both necessary and sufficient to mediate vesicle coat assembly on Golgi membranes. For actin assembly, however, it is necessary but not sufficient, in that GTP γ S is still required for actin assembly even when ARF is bound to the membrane. These results implicate a second GTPase in ARF-mediated actin assembly. Using selective inhibitors of members of the rho-family of GTPases, we provide evidence that ARF acts in concert with a cdc42-dependent signaling pathway to trigger actin polymerization on the Golgi membranes. Elucidating the mechanisms of ARF-sensitive regulation of the cytoskeleton provides insight into the possible roles of the cytoskeleton in Golgi trafficking. This work could also provide a model system for characterizing the mechanisms of regulated actin assembly on biological membranes.

VAC17p, A NOVEL VAC8p-ASSOCIATED PROTEIN,
IS REQUIRED FOR VACUOLE INHERITANCE

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In the yeast *Saccharomyces cerevisiae*, the bud inherits the vacuole from the mother cell. Movement of the vacuole is coordinated with the cell cycle, and requires actin, myosin, and Vac8p, a vacuolar membrane protein. Here we report that Vac17p is also required for vacuole inheritance and movement. Vac17p mediates vacuole inheritance through its interaction with Vac8p. Based on deletion analysis, Vac17p can be divided into four domains: a N-terminal coiled-coil domain, a central PxxP domain, a serine rich domain, and a C-terminal coiled-coil domain. Immunofluorescence studies reveal that Vac17p localizes in dots on the vacuolar membrane. Fractionation analysis confirms that the majority of Vac17p is associated with the vacuolar membrane, although a small portion of Vac17p is soluble. In the $\Delta vac8$ mutant, most of the Vac17p is not on the vacuolar membrane, however, the level of Vac17p is elevated in comparison with that of wild type strains. In contrast, a mutated Vac17p missing the PxxP domain interacts with Vac8p stronger than the wild type Vac17p and localizes to the vacuolar membrane. However, it cannot support vacuole inheritance. These results suggest that Vac8p is one binding receptor for Vac17p. Since PxxP motifs are reported to bind SH3 domain-containing proteins and several actin-related proteins contain SH3-domain(s), we hypothesize that Vac17p assembles the transport machinery on the vacuolar membrane *via* Vac8p and the putative PxxP-binding partner. Studies to identify this partner are in progress.

DISRUPTION OF GOLGI STRUCTURE AND FUNCTION IN MAMMALIAN CELLS EXPRESSING A MUTANT DYNAMIN

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The large GTPase dynamin is a mechanoenzyme that participates in the scission of nascent vesicles from the plasma membrane. Recently, dynamin has been demonstrated to associate with the Golgi apparatus in mammalian cells by morphological and biochemical methods. Additional studies using a well characterized, cell-free assay have supported these findings by demonstrating a requirement for dynamin function in the formation of clathrin-coated, and non-clathrin-coated vesicles from the trans-Golgi network (TGN). In this study, we tested if dynamin participates in Golgi function in living cells through the expression of a dominant negative dynamin construct (K44A). Cells co-transfected to express this mutant dynamin and a GFP-tagged Golgi resident protein (TGN38) exhibit Golgi structures that are either compacted, vesiculated, or tubulated. Electron microscopy of these mutant cells revealed large numbers of Golgi stacks comprised of highly tubulated cisternae and an extraordinary number of coated vesicle buds. Cells expressing mutant dynamin and GFP-tagged VSVG demonstrated a marked retention (8-11 fold) of the nascent viral G-protein in the Golgi compared to control cells. These observations in living cells are consistent with previous morphological and in vitro studies demonstrating a role for dynamin in the formation of secretory vesicles from the TGN.

GPI-Dependent Protein Trafficking in African Trypanosomes

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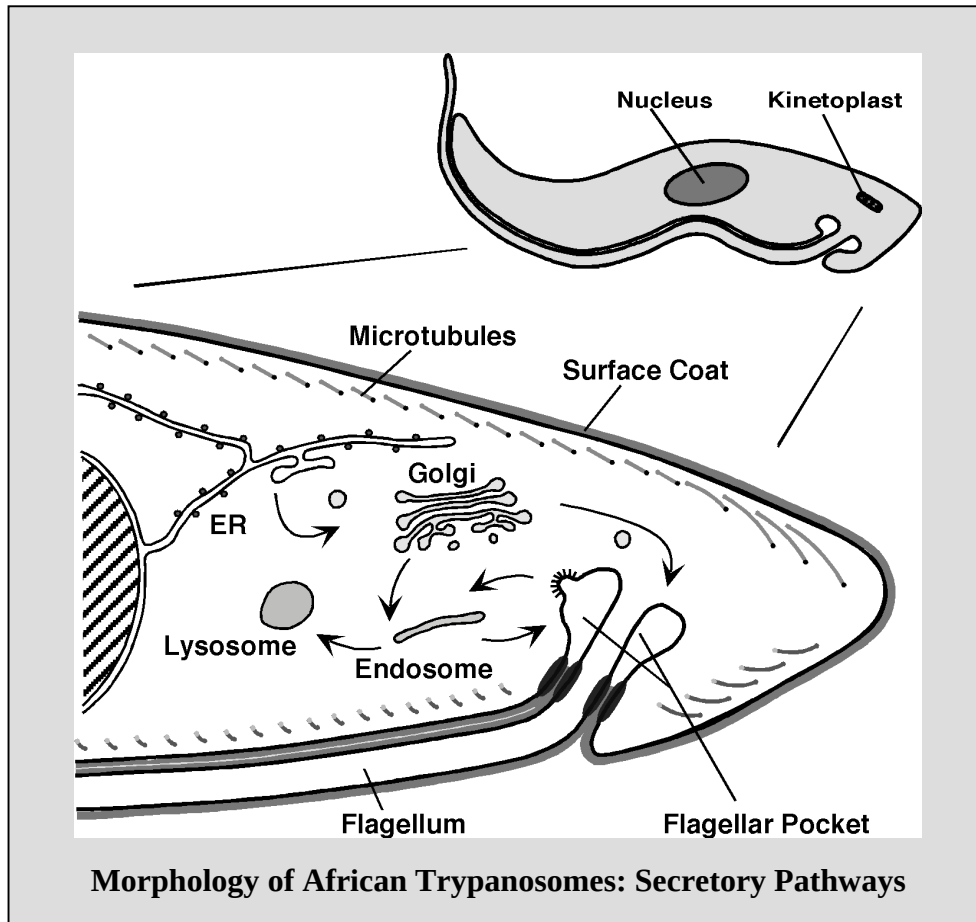
Eukaryotes utilize glycosylphosphatidylinositol (GPI) as an alternative means of attaching proteins to lipid bilayers. Additionally, there is growing evidence suggesting that GPI anchors function in protein trafficking (1). We study the role of this moiety in the secretory trafficking using African trypanosomes (*Trypanosoma brucei*), the protozoan agent of African sleeping sickness. Trypanosomes are $\sim 3 \times 30 \mu\text{m}$ and characteristic eukaryotic secretory organelles (ER, Golgi, lysosomes, etc.) are located between the central nucleus and the posterior flagellar pocket. The organization of sub-pellicular microtubules restricts secretory and endocytic trafficking of macromolecules to this unique subdomain of the plasma membrane. The trypanosomal lifecycle alternates between the midgut of the tsetse fly and the bloodstream of mammalian host. Unique GPI-anchored surface coat proteins are expressed in each stage (2). Procyclin is a protease-resistant monomer that serves a protective function in the hydrolytic environment of the tsetse fly mid-gut. Variant surface glycoprotein (VSG) is a homodimer that is found only in the bloodstream stage. Sequential expression of distinct VSG genes allows the parasite to avoid elimination by the host immune response. As each coat protein is essential and constitutes $\geq 10\%$ of total cell protein, trypanosomes provide an excellent system for studying GPI function.

We have previously investigated the role of GPI anchors in secretory trafficking by heterologous expression of VSG, with and without its GPI attachment peptide, in transgenic procyclic insect stage trypanosomes (3). Deletion of the GPI peptide reduces the rate of VSG transport 5-fold relative to GPI-anchored controls ($t_{1/2} \sim 5$ hrs vs. $t_{1/2} \sim 1$ hr) resulting in an accumulation in the endoplasmic reticulum. This reduced rate does not appear to be due to misfolding because GPI-minus VSG dimerizes efficiently. Furthermore, when co-expressed with GPI-minus VSG, a second soluble reporter, BiPN, is exported rapidly ($t_{1/2} \sim 1$ hr) indicating that there is no general defect in soluble secretion. These results suggest that GPI anchors may act as positive forward transport signals in procyclic trypanosomes.

Here we investigate GPI-dependent trafficking in transgenic bloodstream stage trypanosomes. As in procyclic cells, GPI-minus VSG accumulates in the ER (IFA analysis). Newly synthesized VSG reporter dimerizes efficiently suggesting that retention is not due to quality control mechanisms. Unlike procyclics however, GPI-minus VSG is not secreted but rather is degraded intracellularly. Failure to export this reporter is not due to a general stage-specific reduction in secretion because the soluble BiPN reporter is efficiently exported from transgenic bloodstream cells. Degradation of GPI-minus VSG is partially inhibited by chloroquine and completely blocked by the thiol protease inhibitor P27. IFA of P27-treated cells demonstrates that GPI-minus VSG colocalizes with p67, a lysosomal membrane marker, confirming the lysosome as the site of degradation. These data suggest that in bloodstream trypanosomes GPI anchors function early in the secretory pathway to facilitate ER exit, and then later to mediate proper delivery to the cell surface. The precise location of this latter routing decision is under investigation. Overall our findings indicate that GPI-dependent transport is a general feature of secretory trafficking in both stages of African trypanosomes.

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BOTH CLATHRIN AND PHOSPHATIDYLINOSITOL (3,5) BIS PHOSPHATE CONTROL RETROGRADE TRANSPORT FROM THE YEAST VACUOLE.

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The lysosome is the final destination for many proteins and lipids that undergo endocytosis or autophagy and thus mediates many cellular processes that rely on the appropriate degradation of cellular components. These components arrive *via* multiple membrane trafficking pathways and for these proteins the lysosome is correctly viewed as a “dead end”. However, other proteins that function in the lysosome may recycle back to earlier compartments. To study this process in yeast we have analyzed the targeting of a protein that is shipped to the vacuole via the AP-3 adaptor pathway yet recycles out of the vacuole to reside in the late Golgi.

Previously, we have found recycling out of the vacuole is deficient in *vac7* mutants that are known to have drastically lowered levels of the novel phospholipid, PtdIns(3,5)P₂. Importantly, the recycling of proteins from the late endosome to the Golgi is normal in *vac7* mutants suggesting that these are at least two distinct recycling pathways. We now find that mutations in other genes required for PtdIns(3,5)P₂ levels (*FAB1* and *VAC14*) are also specifically required for vacuolar retrograde traffic but not retrieval of Golgi proteins from endosomal compartments. Thus, PtdIns(3,5)P₂ is an important component in controlling this pathway out of the vacuole. We have now analyzed several candidate proteins in the control vesicle formation at the vacuole and have found a specific role for clathrin in retrograde traffic from the vacuole. Selected alleles of clathrin show a specific defect in vacuolar retrograde transport but are normal for retrieval of Golgi proteins from the prevacuolar/endosomal compartment. These data support a model whereby formation of PtdIns(3,5)P₂ at the vacuole recruits coat proteins such as clathrin to allow for the specific packaging and transport of proteins out of the vacuole.

A NEW COMPONENT OF THE YEAST TRANSPORT SYSTEM

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The cell transport system of the yeast *Sacharomyces cerevisiae* is considerably less complex than the ones in higher eukaryotes, providing of a convenient tool for the elucidation of basic intracellular traffic issues.

Tcp1 (Tailless Complex Polypeptide 1) is a member of a family of about eight paralogs, highly conserved and present in archaea, yeasts, plants, insects, nematodes and mammals. All family members are essential in yeast. The original discovery that linked Tcp1 with the cytoskeleton were easily observable defects in the microtubule organization elicited by a *tcp1* mutant (1). Further connections with the actin cytoskeleton were elucidated in (2, 3). Tcp1 is a microtubule-associated protein that also binds to secretory vesicles. Mutations in the *TCP1* gene lead to abnormalities in nuclear movement and delivery of cell components.

Mutations in the microtubule and actin motors Kar3 and Smy1 and the class V myosin Myo2 resulted in synergistic growth impairments, whereas lack of another kinesin-like motor Cin8 or the dynein Dyn1 had no effect on growth in a *tcp1* mutant background. Hence, mutations in *tcp1* exhibit distinct phenotypes with mutations in different motor proteins. Absence of Kar3 and Myo2 enhanced some of the *tcp1* mutant phenotypes, including defects in cytoskeleton and cell wall organization and cell separation and budding. A *tcp1* mutant exhibited abnormal vacuolar structures, with defects similar to mutants affecting targeting of many vacuolar proteins, endocytosis and retrograde transport.

Tcp1 affects the cytoskeleton, the vesicle transport system, cell wall organization and the signaling system that controls actin and microtubular functions. Its properties indicate that Tcp1 mediates interactions between actin and microtubule motors and their cargoes.

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PHOSPHATIDYLCHOLINE FLIP-FLOP ACROSS THE ER MEMBRANE: SPECIFIC PROTEINS ARE REQUIRED

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A long-standing problem in understanding the mechanism of assembly of the phospholipid bilayer membranes concerns how phospholipids flip back and forth between the two leaflets of the bilayer. of biological meThe question is important because phospholipid biosynthetic enzymes typically face the cytosol and deposit newly synthesized phospholipids in the cytosolic leaflet of biogenic membranes such as the endoplasmic reticulum (ER). These lipids must be transported across the bilayer to populate the exoplasmic leaflet for membrane growth. Transport does not occur spontaneously and it is presumed that specific membrane proteins, flippases, are responsible for phospholipid flip-flop. A biogenic membrane flippase has not been identified and there is controversy as to whether proteins are involved at all, whether any membrane protein is sufficient, or whether non-bilayer arrangements of lipids support flip-flop. To test the hypothesis that specific proteins facilitate phospholipid flip-flop in the ER, we reconstituted transport-active proteoliposomes from detergent-solubilized ER vesicles under conditions where protein-free liposomes containing ER lipids were inactive. Transport of a synthetic, water-soluble phosphatidylcholine analog was found to be sensitive to proteolysis and associated with proteins or protein-containing complexes sedimenting operationally at 3.8S. Chromatographic analyses indicated the feasibility of identifying the transporter(s) by protein purification approaches, and raised the possibility that at least two different proteins are able to facilitate transport. Calculations based on a simple reconstitution scenario suggest that the transporters represent ~0.2% of ER membrane proteins. These results clearly show that specific proteins are required to translocate a phosphatidylcholine analogue across the ER membrane. These proteins are likely to be the flippases required to translocate natural phosphatidylcholine and other phospholipids across the ER membrane. The methodology that we describe paves the way for identification of a flippase.(Menon et al., 2000)

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TWO DISTINCT REGIONS IN A YEAST MYOSIN V TAIL DOMAIN ARE REQUIRED FOR THE MOVEMENT OF DIFFERENT CARGOES.

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When cells divide, cytoplasmic organelles are transmitted from mother to daughter cells. In *Saccharomyces cerevisiae*, lysosome (vacuole) inheritance requires elements of the actin cytoskeleton including actin, profilin, and the class V myosin, Myo2p. Class V myosins function in actin-based movement. They attach to cargo through their globular tail domain, however the receptor is unknown. Myo2p interacts with multiple membranes. In addition to the vacuole, it moves secretory vesicles and likely carries other membranous cargo as well. Secretory vesicle targeting is essential for viability. Thus, removal of the *myo2* tail is lethal. However, vacuole inheritance is not required for viability. This difference led to the discovery of two distinct regions in the tail that bind to different cargo. Through random mutagenesis of the globular tail domain, we obtained seven unique single point mutants with defects in vacuole inheritance, but not polarized growth. One of these mutants was *myo2-2* (G1248D), the others clustered to D1297, L1301, N1304, and N1307, amino acids located on one face of a predicted alpha-helix. Conversely, we identified a second region of the Myo2p tail required only for polarized growth. A deletion of amino acids 1459-1491, cannot support growth as the sole *myo2* gene, but supports vacuole movement in *myo2-2* cells. That specific regions of the globular tail are required for different functions of Myo2p predicts the presence of cargo-specific receptors for Myo2p. In order to identify the vacuolar receptor for Myo2p, we screened for both genomic and multicopy suppressors of the *myo2* vacuole inheritance mutants identified above. We have identified one dominant genomic suppressor. We have also identified two open reading frames that partially suppress the *myo2-2* vacuole inheritance defect when overexpressed.

CHARACTERIZATION OF PHOSPHATIDYLSERINE TRANSPORT TO
PHOSPHATIDYLSERINE DECARBOXYLASE 2 IN PERMEABILIZED
YEAST *SACCHAROMYCES CEREVISIAE*

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In yeast, nascent PtdSer can be transported to the mitochondria and Golgi/vacuole for decarboxylation to synthesize PtdEtn. In strains with a *psd1Δ* allele for the mitochondrial PtdSer decarboxylase, the conversion of nascent PtdSer to PtdEtn can serve as an indicator of lipid transport to the locus of PtdSer decarboxylase 2 (PSD2) in the Golgi/vacuole. We have followed the metabolism of [³H]-serine into PtdSer and PtdEtn to study lipid transport in permeabilized *psd1Δ* yeast. PtdSer transport can be reconstituted in a 2 component system using washed, permeabilized *psd1Δ* cells as donors and membranes isolated by ultracentrifugation as acceptors. With this system the decarboxylation of nascent PtdSer is dependent upon the concentration of acceptor membranes. When limited acceptor membranes are present, PtdSer transport can be slightly stimulated by the addition of high-speed supernatant, suggesting that cytosolic factor may also be involved in the transport process. PtdSer transport is sensitive to the presence of EDTA, and requires Mn²⁺ ions. The role of Mn²⁺ in PtdSer transport appears unique as it can not be replaced by other divalent cations. NIH grants: GM19162, GM32453.

REGULATION OF ER/GOLGI SNARE COMPLEX FORMATION *IN VITRO*

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ER/Golgi membrane transport in mammalian cells is controlled in part by a set of interacting SNARE proteins that includes syntaxin 5, membrin, rbet1 and sec22b. To investigate the precise mechanisms by which these proteins function *in vivo*, we have reconstituted SNARE complexes from the purified proteins *in vitro*. Two higher-order SNARE complexes can form *in vitro* from this collection of SNAREs. In the presence of all four proteins, a quaternary complex forms containing syntaxin 5, membrin, rbet1 and sec22b. Here we report on the stoichiometry of the final complex. We also demonstrate that assembly of this complex is negatively regulated by the syntaxin 5 amino terminal region. Others have shown that the N-terminus of the syntaxin Ssop is a kinetic inhibitor of the yeast exocytotic SNARE complex. Hence, it would appear that negative regulation by amino terminal domains is a general property of syntaxins. Interestingly, in the absence of sec22b, an alternative SNARE complex can form involving only syntaxin 5, membrin and rbet1. Sec22b cannot interact with this pre-assembled ternary complex. Apparently the binding site for sec22b is absent or is occupied by a second copy of one of the proteins. The possible function and regulatory importance of this complex is explored. Finally, we demonstrate that SNARE complexes that include both syntaxin 5 and sec22b are indeed present in cells and are not an artifact of overly promiscuous *in vitro* SNARE interactions.

THE DYNAMIN-LIKE PROTEIN (DLP1) TUBULATES MEMBRANE IN VIVO and IN VITRO.

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Dynamins are large GTPases that have been shown to participate in secretory and endocytic vesicle formation through the tubulation and constriction of membrane. This dynamic process is achieved by the assembly of dynamin into a concentric polymer that can constrict upon GTP hydrolysis. Recently we have identified a dynamin-like protein (DLP1) from mammalian tissues that is essential for the proper cellular distribution of mitochondria and the endoplasmic reticulum in cultured cells (Mol. Biol. Cell, 10: 4403-4417, 1999). Currently, it is unclear if DLP1 can polymerize into ring structures to model membrane and function like conventional dynamin. Therefore, the **GOAL** of this study was to test if DLP1 can tubulate membrane in living cells and, using in vitro assays, determine if this tubulation process is also mediated by polymerization events similar to the conventional dynamins. **RESULTS:** We have previously shown that the expression of two mutations in GTP-binding elements of DLP1 (DLP1-K38A and DLP1-D231N) in cultured cells led to altered distributions compared to the wild type (WT). In striking contrast to normal punctate distribution, the DLP1 mutant proteins were either clustered into large cytoplasmic aggregates (K38A) or were diffuse and cytosolic (D231N). These altered localizations suggest that GTP binding and hydrolysis regulate DLP1 distribution. GTP binding and hydrolysis assays demonstrated that D231N is unable to bind GTP while K38A binds but does not hydrolyze or release GTP and therefore is predicted to have a high affinity for membranes. Indeed, electron microscopy of the cells expressing the DLP1-K38A revealed clusters of short membrane tubules. These constricted membrane tubules are remarkably consistent in diameter (30-35nm) and are found to be coated with DLP1 protein by immunoelectron microscopy. Higher magnification revealed the presence of regular striations along individual membrane tubules. To reconstitute this membrane tubulation by DLP1 in vitro, we utilized purified DLP1 in the presence or absence of synthetic liposomes. We found that DLP1 protein alone assembled into sedimentable macromolecular structures in the presence of GTP γ S but not GTP. Electron microscopy of the GTP γ S-treated DLP1 revealed clusters of stacks of helical ring structures. When liposomes were included with DLP1, formation of long membrane tubules were observed. Addition of GTP γ S greatly enhanced membrane tubule formation, suggesting a GTP-dependent mechanochemical activity of DLP1. In **SUMMARY**, these results provide the first evidence that a dynamin-related protein, DLP1, is able to polymerize, tubulate, and constrict membranes both in living cells and in vitro. Further, these findings also indicate that despite the limited homology to conventional dynamins (35%) these proteins function in a similar fashion.

The Ca²⁺-dependent Interaction Between The C Terminus Of SNAP25 And Synaptotagmin Is Essential For Regulated Exocytosis In PC12 Cells

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SNAP25 and synaptotagmin (Syt) are key components in regulated exocytosis. Previously our in vitro studies indicated that there was a Ca²⁺-dependent interaction between the C terminus of SNAP25 and Syt. Here, we provide in vivo evidence that this interaction is essential for regulated exocytosis. We used site-directed mutagenesis to generate a BoNT/E-resistant SNAP25 (D179K) tagged with influenza hemagglutinin (HA) epitope. The D179K/HA mutant functionally replaced endogenous SNAP25 in PC12 cells in transfection studies when a BoNT/E light chain was co-expressed. In contrast, a double mutant harboring a second substitution in the C terminus of SNAP25, D179K/D186K/HA SNAP25, exhibited loss-of-function in regulated exocytosis confirming the importance of the C terminus. Immunoprecipitations with a HA monoclonal antibody were conducted to assess protein associations for the mutant SNAP25 in the transfected cells. The D179K/HA and D179K/D186K/HA SNAP25 associated similarly with syntaxin and VAMP in SNARE complexes. Immunoprecipitated D179K/HA SNAP25 exhibited a Ca²⁺-dependent interaction with Syt whereas this interaction was decreased with the D179K/D186K/HA SNAP25. We conclude that the C terminus of SNAP25 is essential for both regulated exocytosis and for Ca²⁺-dependent interaction with Syt, and propose that the Ca²⁺-dependent Syt-SNAP25 interaction is an essential component of the Ca²⁺ sensor for exocytosis.

Identification of novel pathway for Ras protein trafficking in *Saccharomyces cerevisiae*

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Ras proteins role in signal transduction requires subcellular localization to the inner leaflet of plasma membrane (PM). This is accomplished in part by a series of posttranslational modifications of a CaaX motif, where C is Cys, *a* generally an aliphatic residue and *X* is the C-terminal residue. The modifications include farnesylation, -*aaX* proteolysis, methylation and palmitoylation. However, it is not clear how these modifications contribute to the correct subcellular localization of Ras. We have therefore performed a genetic screen to identify mutants involved in Ras transport in yeast. Mutations in two genes, *ERF2* and *ERF4*, were identified from the screen. *ERF2* encodes a protein with four transmembrane domains and a DHHC-Cys rich domain (1). *ERF4* was previously identified as SHR5 and has been implicated in Ras function, although its role is unknown (2). Both Erf2p and Erf4p are localized to the endoplasmic reticulum (ER) membrane. Genetic and two-hybrid analysis revealed that Erf2p and Erf4p interact directly. A conditionally expressed GFP-Ras2 fusion protein was constructed to examine the subcellular localization of Ras in wild type and mutant strains. Using a set of temperature sensitive secretory mutants (*sec23*, *sec14*, *sec9*), we have shown that the conventional secretory pathway is not required for Ras localization to the PM (3). However, deletion of either *ERF2* or *ERF4* along with *sec23* or *sec14* blocks PM localization of GFP-Ras. These results suggest that Erf2p and Erf4p work together as part of a novel pathway for translocation of Ras proteins from the ER to the PM.

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