The Exocyst Affects Protein Synthesis by Acting on the Translocation Machinery of the Endoplasmic Reticulum

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Summary

We previously showed that the exocyst complex specifically affected the synthesis and delivery of secretory and basolateral plasma membrane proteins. Significantly, the entire spectrum of secreted proteins was increased when the human Sec10 (hSec10) component of the exocyst complex was overexpressed, indicative of post-transcriptional regulation (1). Here, using an exogenous transfected basolateral protein, the polymeric immunoglobulin receptor (pIgR), and a secretory protein, gp80, we show that pIgR and gp80 protein synthesis and delivery are increased in cells overexpressing Sec10 despite the fact that mRNA levels are unchanged, highly suggestive of post-transcriptional regulation. To test specificity, we also examined the synthesis and delivery of an exogenous apical protein, concentrative nucleoside transporter 1 (CNT1), and found no increase in CNT1 protein synthesis, delivery, or mRNA levels in cells overexpressing Sec10. Sec10-GFP overexpressing cell lines were created and staining is seen in the endoplasmic reticulum (ER). It was previously demonstrated in yeast that high levels of expression of SEB1, the Sec61β homologue, suppressed sec15-1, an exocyst mutant (2). Sec61β is a member of the Sec61 heterotrimer, which is the main component of the ER translocon. By co-immunoprecipitation we show that Sec10, which forms an exocyst subcomplex with Sec15, specifically associates with the Sec61β component of the translocon and that Sec10 overexpression increases the association of other exocyst complex members with Sec61β. Proteosome inhibition is not the mechanism by which we find increased protein synthesis in the face of equivalent amounts of mRNA. Though the exact mechanism remains to be elucidated, the exocyst/Sec61β interaction represents an important link between the cellular membrane trafficking and protein synthetic machinery.

Introduction

Proteins destined for secretion, or delivery to the plasma membrane, are translocated across the rough endoplasmic reticulum (RER) via the translocon in eukaryotic cells. After modification and folding, these proteins are then transported in vesicular carriers to the Golgi complex, and trans-Golgi network (TGN). From the TGN proteins can be sorted to several sites, including lysosomes, and to the plasma membrane for insertion or release into the extracellular space (3,4). There are several possible ways in which traffic through the secretory pathway is regulated. One of the best understood is the quality control machinery in the RER and Golgi. Proteins must properly fold and assemble before they can be efficiently exported. The rate and extent to which certain proteins reach the cell surface or are secreted is controlled by this mechanism (5). A second type of regulation is in dense core secretory granules. In the TGN of
cells that contain such granules, certain proteins are selectively sorted into the granules, where they are stored. Upon receipt of an extracellular signal and elevation of intracellular free calcium, these granules can rapidly fuse with the plasma membrane, leading to the release of their contents (6). With the exception of these regulatory mechanisms, traffic through the secretory pathway is thought to proceed in a relatively constitutive manner. The amounts of individual proteins that reach the cell surface, or are secreted, has therefore been thought to be primarily regulated at the level of mRNA transcription, rather than at the level of the secretory pathway.

Polarized epithelial cells have two distinct plasma membrane domains with very different protein compositions and functions: an apical domain comprising the luminal surface, and a basolateral domain contacting adjoining and underlying cells. To establish and maintain their polarity, epithelial cells synthesize and then deliver specific proteins to the correct apical or basolateral plasma membrane (7). Madin-Darby canine kidney (MDCK) cells represent one of the best-studied polarized epithelial cell lines and are derived from canine renal tubular epithelium (8). When grown on a permeable filter, MDCK cells form a well-polarized epithelial monolayer, exhibiting apical and basolateral plasma membrane domains with unique compositions (9,10), and well-defined cell-cell junctional complexes containing tight junctions (11).

There are circumstances where the cell might need to regulate the level of whole classes of proteins, such as those found in the plasma membrane, or in particular in the basolateral plasma membrane of polarized epithelial cells. For example, cells must often alter their shape, polarization, and other characteristics during development and differentiation, and this plasticity could require the coordinate regulation of large classes of proteins. We have experience with two in vitro models where polarized epithelial cells coordinately regulate the abundance of all of the proteins at their basolateral plasma membrane. First, when these cells are dissociated by trypsin and EDTA and then plated in culture, the cells are initially relatively flat. As the cells establish contacts and become confluent, they become columnar and their lateral surfaces increase greatly. This process resembles the polarization of epithelial cells that occurs during normal development. Second, when MDCK cells are grown in three-dimensional collagen gels, they form hollow cysts lined by a monolayer of cells with their apical surfaces facing the center (12). Addition of hepatocyte growth factor (HGF) causes the cells to first send out long extensions of their basolateral plasma membrane, which requires an increase in basolateral surface area. Eventually, cells migrate out and reorganize into tubules in a process resembling tubulogenesis in vivo (13,14).
One candidate for a component of the machinery in this process is the exocyst (a.k.a. sec6/8) complex, first identified as being involved in the exocytosis of vesicles in yeast (15). The exocyst is a 750Kd complex comprised of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (16,17). In yeast (and probably mammals), Sec10p and Sec15p exist as a subcomplex within the greater exocyst complex. This Sec10/15 subcomplex acts as a bridge between the rab GTPase Sec4p, found on the surface of the secretory vesicles carrying polarized proteins, and the rest of the exocyst complex, which is in contact with the plasma membrane (18). In permeabilized MDCK cells, antibodies to the Sec6 and Sec8 subunits inhibit delivery of at least one basolateral protein to this surface but do not affect the delivery of at least one apical protein to the apical surface (19).

We have previously reported that overexpression of the human homologue of Sec10, hSec10, in MDCK cells resulted in multiple effects: (i). Polarized hSec10 overexpressing cells grown on a two-dimensional Transwell filters were significantly taller, but not wider, than control cells, suggesting an increased basolateral surface area and protein delivery. (ii). hSec10 overexpression specifically resulted in increased synthesis and delivery of endogenous basolateral plasma membrane proteins as well as secretory proteins, compared to control cells, as determined by pulse-chase experiments on filter-grown cells. (iii). hSec10 overexpressing cells in a three-dimensional collagen gel system formed cysts much more rapidly and efficiently than did control cells. When these cysts were stimulated with HGF, they produced more tubules than did cysts made up of control cells (1).

These pleiotropic morphologic responses probably reflect an underlying increase in the synthesis of endogenous basolateral plasma membrane proteins and the consequent expansion of the basolateral surface area. We are less certain of the consequences of the increase in synthesis of the entire spectrum of major secretory proteins that are released into the apical and basolateral media. Our initial interpretation of these data was that, most likely, the increase in production of secretory and basolateral plasma membrane proteins was produced by the coordinated transcriptional up-regulation of all of these proteins. However, because these proteins have very different functions and are probably under the control of diverse transcriptional regulatory networks, this argues against a transcriptional model. For instance, the major secretory protein in MDCK cells is gp80 (20), a protein with 80% homology to rat-sulfated glycoprotein 2 (SGP-2). SGP-2 is the major protein secreted by rat Sertoli cells and is presumably under complex hormonal control (21,22). What these basolateral plasma membrane and secretory proteins have in common, however, is they all transit through the secretory pathway. Regulation of their
transit through the secretory pathway could be a facile means of regulating the abundance of these proteins.

The translocon, or protein-conducting pore in the RER through which newly-made proteins are translocated into or across the RER membrane, consists of three main subunits, Sec61α, β, and γ (23-25). While the Sec61α subunit is primarily responsible for translocation, much less is known about the Sec61β subunit. It is not required for translocation of all proteins in reconstituted liposomes or for viability in yeast, but it does facilitate translocation (26). Seb1p is a yeast homologue of Sec61β. Intriguingly, SEB1 was isolated as a multicopy suppressor of the sec15-1 mutation, and, given that Sec15 is a component of the exocyst complex, this implies at least a genetic interaction of the exocyst and translocon (2).

Here we test the hypothesis that the exocyst is involved in the regulation of traffic through the secretory pathway, especially in coordination of events at the plasma membrane with events earlier in the secretory pathway, specifically those at the translocon. We present data showing that exocyst overexpression, despite unchanged mRNA levels, increases synthesis of basolateral and secretory, but not apical, proteins and biochemical evidence that the exocyst associates with Sec61β. We propose that this association is one mechanism by which exocyst overexpression leads to an increase in secretory and basolateral protein synthesis.

**Experimental Procedures**

**Cell culture**

MDCK type II cells overexpressing hSec10 (1) and control MDCK cells were maintained in MEM-containing Earle's balanced salt solution, supplemented with 5% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO2/95% air.

**Antibodies/Reagents**

Monoclonal mouse anti-rSec6 and -8 was used at 1:500 for Western blot and 1:100 for immunofluorescence (StressGen). IP3R3 monoclonal antibody was used at 1:100 for immunofluorescence (Transduction Laboratories). Monoclonal anti-myc antibodies were used at 1:1000 for Western blot. Sec61β antibodies were used at 1:500 for Western blot. For immunofluorescence Alexa 594 secondary antibody was used at 1:200 (Jackson ImmunoResearch). Goat anti-mouse HRP (Jackson Labs) was the secondary antibody used for Western blots (1:15,000) (Jackson ImmunoResearch).
Northern Blot

MDCK cells overexpressing Sec10 and pIgR or just pIgR were grown on Transwell filters for 6-7 days as described (27). RNA was then isolated using RNA STAT-50 (Tel-Test Inc.). The RNA was transferred to a nylon membrane and blotted in standard fashion (28) using 32P-labeled pIgR and GAPDH cDNA probes generated with “Prime-a-Gene” (Promega). cDNA probes for CNT1 and gp80 (a kind gift from Dr. Claudia Koch-Brandt) were used in a similar fashion for their respective Northern blots.

Adenovirus Production

The tetracycline regulated promoter has been subcloned from the tetracycline inducible expression plasmid pUHD10-3 to pAdlox (29) 3' to the ψ5 packaging site and 5' to the polyA site, replacing the original CMV promoter with the regulated tetracycline promoter (30). Full-length hSec10 was then cloned into the adenovirus vector just downstream of the tetracycline/minimal CMV promoter. In the presence of doxycycline, a tetracycline derivative, expression of hSec10 is tightly repressed.

To examine function of the adenoviral constructs a Western blot was performed using antibodies against the myc epitope tag, which confirmed hSec10-myc overexpression. As a further validation of both the function of our recombinant adenovirus and the experiment shown in Fig. 1a, increased pIgR protein synthesis was seen with an 35S-methionine pulse in cells infected with just hSec10-myc adenovirus and tetracycline transactivator (ttt) adenovirus compared to control cells infected with hSec10-myc adenovirus, tta adenovirus, and doxycycline (data not shown).

Generation of Sec10-GFP and Transfection

The cDNA coding for hSec10 was cloned into the Enhanced Fluorescent Protein (EGFP) vector (Clontech) and then transfected into MDCK cells using a calcium-phosphate precipitation method as previously described (1,27). Selection was performed using medium containing G418 (Gibco).

Synthesis, Delivery, and Secretion Assays

MDCK type II cells constitutively expressing the basolateral protein pIgR and either overexpressing hSec10 or an empty vector from a confluent 100 mm plastic dish were trypsinized and 5% of the cells in the dish were seeded per 12 mm (0.4 um pore size) Transwell filter (Costar). The cells were grown for 6-7 days with fresh medium added daily and allowed to grow to confluence as determined by hydrostatic pressure testing (27). After washing with PBS, cells were starved for 20 minutes in MEM medium lacking methionine and then labeled by...
exposing the basolateral surface to a 25 ul drop of starvation medium containing 4 ul of $^{35}$S-methionine (31.4 uCi/ul, NEN) for 20 minutes. After pulsing with $^{35}$S-methionine for 20 minutes, cells were lysed in 0.5% SDS, equal volumes of 2.5% Triton X-100 were added, and immunoprecipitation was performed using antibody against pIgR. The immunoprecipitate was then run on an SDS-PAGE gel and analyzed with a phosphorimager (Molecular Dynamics).

For the synthesis and delivery assays of the apical plasma membrane protein CNT1, MDCK cells constitutively overexpressing pIgR and CNT1-GFP were grown on 12 mm Transwell filters for 6 days. 24 hours before the pulse chase was performed all the Transwells received 3 ul of tta and 3ul of Sec10 virus stock corresponding to approximately 15 plaque-forming units/cell of recombinant adenovirus in 2 ml of binding media (Hank's salt solution containing 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 10 mM Hepes, pH 7.2) for 1 hour at 37$^\circ$C to enable viral attachment and infection as described (30). Half of the Transwell filters also received doxycyline at 20 ng/ml to repress expression of Sec10. The synthesis and delivery assays were then performed as described above using anti-GFP antibody (Roche) to immunoprecipitate CNT1-GFP.

For the secretion/proteosome inhibitor assays, half the Transwell filters containing hSec10-overexpressing cells were treated with the proteosome inhibitor MG132 (Calbiochem) for one hour at 25 uM as previously described (31). The cells were labeled as described above, washed extensively, and MEM medium was added (0.3 ml apically and 0.5 ml basolaterally) and collected at sixty minutes, run on an SDS-PAGE gel, and analyzed with a phosphorimager (Molecular Dynamics). All experiments were repeated at least three times and were reproducible.

**Western Blot/Co-Immunoprecipitation**

Cells were lysed in 1% N-octylglucosid (Boehringer-Mannheim) and prepared in standard fashion (32). Immunoprecipitation was performed using 4-8 ug of antibody against myc, Sec6, Sec8, or Sec61$\beta$ per immunoprecipitation. The immunoprecipitate was then run on an SDS-PAGE gel. The protein bands were detected by incubations with the same myc antibody (1:500) or Sec61$\beta$ (1:500) followed by goat anti-mouse HRP (Jackson Labs) as the secondary antibody (1:15,000), and ECL (NEN).

**Immunofluorescence and Confocal Microscopy**

Cells were rinsed in PBS and fixed for 30 minutes with 4% paraformaldehyde as previously described (1,27). Nonspecific binding sites were blocked and the cells permeabilized using 0.7% fish skin gelatin and 0.025% saponin. Samples were placed in medium containing antibody to IP$_3$R3 (Transduction Labs). After extensive washing, the samples were incubated in
blocking buffer containing Alexa 594-conjugated secondary antibody, 1:200 dilution (Molecular Probes). Cells were postfixed with 4% paraformaldehyde and mounted. Confocal images were collected using a krypton-argon laser (Biorad 1024).

**Statistics**

Unitless phosphorimager counts in Figures 1 and 6 were significant within an experiment but not between experiments. For all statistics reported, the average and standard deviation were performed using Excel (Microsoft) software.

**Results**

**Overexpression of hSec10 Causes Increased Synthesis of Exogenous Basolateral, but not Apical, Proteins**

We have previously shown that hSec10 overexpression increased synthesis and delivery of all major secretory proteins and the endogenous basolateral protein E-Cadherin, but not the endogenous apical protein gp135 (1). Other groups, while not looking at synthesis or secretion, have shown that the exocyst specifically affects basolateral protein delivery to the cell surface (19,33,34). As noted, the fact that all the major secretory proteins were increased (and by approximately the same degree) made a coordinated transcriptional effect seem unlikely, given the diversity of transcriptional regulation.

As a first step to test if the effect of hSec10 on synthesis of basolateral plasma membrane proteins was transcriptional or post-transcriptional, a plasmid encoding the cDNA for an exogenous basolateral protein, the polymeric immunoglobulin receptor (pIgR), driven off of a cytomegalovirus (CMV) promoter, was transfected into the MDCK parent cell line prior to transfection of hSec10. We found increased synthesis of the exogenous basolateral pIgR in the hSec10 overexpressing compared to control cells (Fig. 1a). We also found increased pIgR protein delivery to the basolateral plasma membrane at a degree corresponding to the increase in protein synthesis (data not shown). This cDNA was engineered to contain minimal 5' and 3' untranslated regions (no introns or likely regulatory elements) (35). The CMV promoter itself is a very strong and constitutively active promoter in MDCK cells (27). There is no a priori reason to suspect that the CMV promoter should be regulated by hSec10 or the exocyst.

We took the same MDCK cells, stably overexpressing pIgR, and previously transfected them with a pcDNA3 plasmid, which also utilizes the CMV promoter, containing cDNA
encoding the rat concentrative nucleoside transporter 1 (CNT1), an apical protein. Stable cell lines expressing CNT1 with a green fluorescence protein (GFP) tag were created and it was confirmed that CNT1-GFP localized to the apical plasma membrane and that the GFP tag did not alter substrate selectivity (36). This cell line constitutively expressing the apical CNT1-GFP allowed us to do a similar experiment to the one described above for the basolateral protein pIgR. However, to do this experiment we used the approach of infecting the cells with a recombinant adenovirus encoding hSec10. The adenoviral approach was necessary because we were limited by the number of selection markers that could be used for transfection in this cell line (i.e. the CNT1-GFP cell line had already been transfected with plasmids encoding pIgR and CNT1-GFP). The recombinant adenovirus was constructed so that the hSec10 cDNA was driven off of a minimal CMV promoter which itself was fused to a regulated tetracycline promoter. Expression of hSec10 was, therefore, repressed in the presence of doxycycline, a tetracycline derivative. Tests confirming the function of the recombinant adenoviral construct were performed (see methods). The cells were grown on Transwell filters and, twenty-four hours prior to the 35S-methionine pulse experiments, were infected with recombinant adenovirus encoding hSec10, adenovirus encoding the tetracycline transactivator (tta), and, in half the cases, doxycycline to suppress hSec10 expression. We found no increase in CNT1 protein synthesis in cells that overexpressed hSec10 (Fig. 1b) consistent with an exocyst effect (1,19,33,34). There was also no significant increase in CNT1-GFP delivery to the apical surface in cells overexpressing hSec10 (data not shown).

Assuming the pCB7 plasmid containing pIgR cDNA was constitutively active, pIgR mRNA should have been present in similar amounts in both control and hSec10 overexpressing cells. Northern blot analysis of pIgR mRNA from control and hSec10 overexpressing cells confirmed that mRNA levels were unchanged in spite of the increased protein synthesis (Fig. 2a). Similarly, we had previously shown that there was increased synthesis of the secretory protein gp80 in hSec10-overexpressing cells (1). Here we show by Northern blot that despite increased synthesis of gp80, there was no increase in gp80 mRNA levels (Fig. 2b). We also showed above that there was no increase in the synthesis of the apical protein CNT1 in cells overexpressing hSec10. There was also no significant change in CNT1 mRNA levels (Fig. 2c). This indicated to us that hSec10 overexpression did not alter the steady state level of pIgR and gp80 mRNA, and most likely changed either the rate of synthesis or degradation of pIgR and gp80 by acting at a step downstream of the level of mRNA.

Overexpressed hSec10-GFP Partially Localizes to the ER by Immunofluorescence and Co-immunoprecipitates with Sec61β
As noted, high-level expression of the translocon subunit Sec61β suppressed a mutant of Sec15 in yeast (2). Knowing that Sec10p and Sec15p form a subcomplex within the greater exocyst complex (18), this suppression suggested an interaction of part of the exocyst, particularly the Sec10/Sec15 subcomplex, with the translocation machinery. If true, some portion of the hSec10 should localize to the RER. We cloned hSec10 into the Enhanced Fluorescent Protein (EGFP) vector (Clontech) and looked at expression of hSec10-GFP in polarized MDCK cells grown on Transwell filters. At least for cells containing the highest expression of hSec10-GFP, we saw partial co-localization of hSec10-GFP with the ER resident protein, type 3 inositol 1,4,5-triphosphate receptor (IP3R3) (Fig. 3) (it is possible that in cells with lower levels of hSec10-GFP, the specific signal of hSec10 in the ER is not detectable above the diffuse cytosolic background, which is characteristic of transfected chimeras of GFP and exocyst subunits (37)). This placed the exocyst complex in an area where it could be involved in the process of protein translocation and/or translation and was similar to results from Shin and colleagues who co-localized the endogenous exocyst with IP3R3 in pancreatic acinar cells (38).

We therefore looked for a physical interaction between the transfected hSec10, containing a myc epitope tag (hSec10-myc), and Sec61β proteins. We solubilized MDCK cells expressing both proteins, tested for co-immunoprecipitation of hSec10-myc with Sec61β, and found that hSec10-myc and Sec61β specifically co-immunoprecipitated. This co-immunoprecipitation could be observed in both directions, i.e. by immunoprecipitation of Sec61β and looking for co-immunoprecipitated hSec10-myc, and, conversely, by immunoprecipitation of hSec10-myc and looking for co-immunoprecipitated Sec61β (Fig. 4a, d). Per 12 mm Transwell filter, there was an equivalent amount of Sec61β in hSec10-myc overexpressing and control cells (Fig. 4c). By quantitating the intensity of the hSec10-myc band from a 10 ul aliquot of the hSec10-myc cell lysate (Western blot only, lane ‘M’, Fig. 4a) and comparing that to the intensity of the hSec10-myc band that co-immunoprecipitated from 750 ul of total lysate, itself immunoprecipitated with Sec61β (lane ‘S’, Fig. 4a), and adjusting for inefficient or incomplete immunoprecipitation (Fig. 4b), we estimate that ~35% of total cellular hSec10-myc co-immunoprecipitated with the Sec61β.

We adjusted for inefficient or incomplete immunoprecipitation, i.e. the amount of protein that is lost during an immunoprecipitation, by immunoprecipitating hSec10-myc from 750 ul of total cell lysate using antibody against the myc-epitope tag and then blotting for myc. This band was then compared to the band from 10 ul of lysate (Western blot only). We found that instead of the expected 75-fold (750ul/10ul) amount of hSec10-myc protein, assuming 100% of the...
hSec10-myc protein was immunoprecipitated, there was only ~11-fold (Fig. 4b). This was very reproducible for hSec10-myc, though when a similar exercise was done with Sec61β (i.e. immunoprecipitate 750 ul of total lysate with Sec61β antibody and then blot for Sec61β, Fig. 4c), instead of the expected 75-fold excess there was only ~2-fold. We attribute the ~5-fold variation in efficiency of immunoprecipitation between hSec10-myc and Sec61β to differences in the respective antibodies. Immunoprecipitation was performed using 4 ug of antibody against myc or Sec61β per immunoprecipitation. Increasing the amounts of myc and Sec61β antibody up to 15 ug did not increase the amount of myc and Sec61β protein co-immunoprecipitating beyond what was seen in Figures 4b,c. Using similar calculations, we estimate that ~7.5% of the total Sec61β co-immunoprecipitated with hSec10myc (Fig. 4c,d), though it should be noted that endogenous Sec10 is also binding Sec61β to a degree that we could not quantify. In addition, as controls we used a panel of antibodies to ER proteins such as SSR and GP94; all showed a lack of co-immunoprecipitation, corroborating the specificity of the interaction between hSec10-myc and Sec61β (Fig. 4e).

In hSec10 overexpressing cells, it is possible that hSec10 could be acting on the ER translation machinery on its own or in combination with Sec15, as the Sec10/Sec15 subcomplex has been shown to migrate separately from the rest of the exocyst complex (18). hSec10 could also be acting through the entire exocyst complex. In order to examine the role of the other exocyst complex members in this process, we used antibodies against endogenous Sec6p and Sec8p in co-immunoprecipitation experiments. We show that endogenous Sec6p and Sec8p also co-immunoprecipitate with Sec61β, ~6% and ~2% respectively. Furthermore, using cell lysates from hSec10 overexpressing and control cells, we found that overexpression of hSec10 increased the amount of Sec61β that co-immunoprecipitated with the endogenous exocyst complex members Sec6p and Sec8p, now ~13% and ~6% respectively (Fig. 5). This could suggest a direct interaction between the entire exocyst complex and Sec61β or, alternatively, with increasing amounts of hSec10, an indirect association of Sec6 and Sec8 with Sec61β is strengthened, perhaps by simple mass action.

Effect of Proteosome Inhibition on Secreted Proteins

Given the striking increase in secretory and basolateral plasma membrane protein levels in cells that overexpress hSec10, one explanation could be decreased degradation of newly made proteins in addition to, or exclusive of, increased protein synthesis. Proteins are degraded inside a cell for many reasons including failure of the proteins to assemble into a complex or the existence of proteins in a damaged or misfolded state. Such proteins are exported from the ER
back into the cytosol, where they are degraded. This retrotranslocation, also called dislocation, occurs via the same Sec61 translocon through which the proteins initially entered the ER, although additional proteins help the Sec61 complex act in reverse. Sec61β has been shown to play a major role in this process of retrotranslocation (39). The major pathway for protein degradation involves marking the proteins for destruction by covalent attachment of a small protein, ubiquitin, before transport to the proteosome complex where proteolysis occurs (40). Since a large fraction of all newly made proteins are immediately degraded, a reduction in this constitutive degradation would result in an “apparent“ (but false) increase in synthesis. In order to test this possibility, we performed pulse-chase experiments using hSec10 cells grown on Transwell filters. Half the Transwell filters were treated with the cell-permeable proteosome inhibitor MG132 (31), and the apical and basolateral media were then collected following the pulse-chase. There was no difference detected in the secretion of gp80, the most abundant secretory protein in MDCK cells (20), or other secretory proteins in hSec10 overexpressing cells treated with proteosome inhibitor compared to the untreated hSec10 overexpressing cells (Fig. 6).

Discussion

Our findings represent a link, in mammals, between the last step of the secretory pathway, exocytosis, and the first step, synthesis of membrane and secretory proteins in the ER. Review of the literature demonstrated at least two precedents for the interaction of the membrane trafficking and protein synthetic machinery. First, in Tetrahymena there is increased synthesis of dense core granule components after degranulation, which could be either a transcriptional or post-transcriptional event (41). Second, in yeast, overexpression of the Sso t-SNARE, which, like the exocyst, is part of the exocytic apparatus, caused increased synthesis of an exogenous bacterial secretory protein (42). This is more likely to be a post-transcriptional event as the bacterial secretory protein was under the control of a constitutively active promoter, and, therefore, was equally active in control and Sso overexpressing yeast.

As noted, several reports and experiments helped us in our search for this connection. First, in yeast, high-level expression of the Sec61β homologue, SEB1, suppressed an exocyst mutant, sec15-1 (2). Using co-immunoprecipitation experiments, we show that the mammalian exocyst complex and Sec61β specifically interact. Second, the exocyst was initially found to localize to the ER where translocation occurs (38), which we confirmed by partial co-localization of overexpressed hSec10-GFP and the ER resident protein IP₃R3. Third, in addition to all major endogenous secretory proteins being increased, synthesis of an exogenous basolateral protein
pIgR, but not an exogenous apical protein CNT1, was increased in hSec10 overexpressing compared to control cells. Finally, Northern blot analysis of mRNA from endogenous gp80 and transfected exogenous pIgR demonstrated that, in spite of the increase in gp80 and pIgR protein synthesis, there was no change in mRNA levels. Taken together, these data indicate that the exocyst was acting to affect protein translation and, or, translocation, which are tightly coupled in the ER. This mechanism suggests that cells selectively control secretory and basolateral plasma membrane protein synthesis as a group not at the level of the nucleus, but, rather, at the first specific stage of their synthetic pathway (i.e., at the level of the translational machinery and translocon of the ER).

Given that proteosome inhibition did not affect secretion in hSec10 overexpressing cells, it is unlikely that exocyst overexpression was simply decreasing the rate of protein degradation. It has previously been shown that the translocon is dynamic and can be regulated (43) and that Sec61β is not essential for co-translational translocation but does kinetically facilitate it (26). Gruss and colleagues showed that calcium dependent isoforms of protein kinase C (PKC) are associated with the rough ER and phosphorylate essential components of the protein translocation machinery including Sec61β. Sec61β is extensively phosphorylated and phosphorylation increased translocation of preprolactin (4). This may be important given that the exocyst has recently been shown to govern the polarized expression of calcium signaling complexes and regulation of their activity in the ER of pancreatic acinar cells (38).

Another possible mode of interaction between the exocyst and Sec61β involves the ribosomal machinery of the cell. In mammalian cells, the import of proteins into the ER begins before the polypeptide chain is completely synthesized--that is, it is a co-translational process. The ribosome that is synthesizing the protein is tightly bound to the ER membrane. Since many ribosomes can bind to a single mRNA molecule, another mechanism for increasing protein synthesis, in the face of a constant level of mRNA, could be to increase the number of ribosomes binding to each mRNA molecule (44). Along similar lines, the Sec61p complex forms the core element of the translocon in the ER membrane. Ribosomes (both translating and non-translating) bind with high affinity to ER membranes that have been stripped of ribosomes or to liposomes containing purified Sec61p (26). Therefore, Sec61p is believed to serve as both a translocation pore and a ribosome-binding site and it has been shown that the Sec61β subunit of the Sec61 complex makes contact with non-translating ribosomes (45). Given our co-immunoprecipitation data showing a specific interaction between Sec61β and hSec10, it is possible that the exocyst complex modulates the interaction between Sec61β and non-translating ribosomes.
In summary, we have demonstrated a link in mammalian cells between the membrane trafficking and protein synthetic machinery. This link could be significant for human diseases involving changes in protein synthesis and polarity such as autosomal dominant polycystic kidney disease (ADPKD) in which the exocyst has been shown to be misexpressed (34). Further studies are needed to determine the exact mechanism by which the exocyst/Sec61β interaction affects protein synthesis.
References

Figure Legends

**Figure 1:** Apical and basolateral protein synthesis as determined by pulse-labeling. a) hSec10-overexpressing and control cells, both stably expressing the basolateral protein pIgR, were metabolically pulse-labeled for fifteen minutes with $^{35}$S-cysteine. The cells were lysed and immunoprecipitation was performed with antibodies against the polymeric immunoglobulin receptor (pIgR). The immunoprecipitate was analyzed by SDS-10% PAGE and radioactivity associated with pIgR was determined using a phosphorimager. Protein synthesis of pIgR was significantly increased in the hSec10 overexpressing compared to control cells. Quantification of the transfected pIgR protein levels in the experiments gave the following results: in hSec10 cells-10,949 +/- 1,101 and in control cells-6,005 +/- 1,405 (p<0.05) arbitrary phosphorimager units. b) MDCK cells stably overexpressing the apical protein CNT1-GFP were infected with adenovirus encoding hSec10, adenovirus encoding the tetracycline transactivator, and in half the cases doxycycline at 20 ng/ml (control) 24 hours prior to performing a pulse with $^{35}$S-methionine. The cells were lysed and immunoprecipitation of the CNT1-GFP fusion protein was performed with antibodies against GFP (as suitable antibodies to CNT1 were not available). The immunoprecipitate was analyzed by SDS-10% PAGE and radioactivity associated with CNT1-GFP was determined using a phosphorimager. There was no significant difference in the amount of CNT1-GFP synthesized in the cells expressing hSec10 compared to the control cells: in hSec10 cells-319,970 +/- 141,066 and in control cells-297,251 +/- 82,627 arbitrary phosphorimager units. NS=not significant. M=14C markers.

**Figure 2:** Northern blot analysis examining mRNA expression in hSec10 overexpressing and control cells. a) The hSec10 overexpressing and control cells were grown on Transwell filters for seven days. The filter-grown cells were then lysed and the mRNA isolated. Nine ug of mRNA was loaded into each well of a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and then probed with $^{32}$P-labeled pIgR cDNA according to standard protocols (28). The membranes were also probed with a “housekeeping” cDNA, GAPDH, to assure that equal amounts of mRNA were loaded into each well. No difference in the amount of pIgR mRNA was seen in the hSec10 overexpressing compared to control cells. Note that no pIgR expression is seen in non-transfected MDCK cells. b) hSec10 overexpressing and control cells were grown as described above and then lysed and the mRNA isolated. Nine ug of mRNA was loaded into each well, transferred to a nylon membrane, and then probed with $^{32}$P-labeled gp80 cDNA. There was no difference in the amount of gp80 mRNA in the hSec10-overexpressing versus control cells. There is expression of endogenous gp80 in all the cell lines. c) MDCK cells stably overexpressing the apical protein CNT1-GFP were infected with adenovirus containing hSec10,
adenovirus containing the tetracycline transactivator, and in half the cases doxycycline (control) 24 hours prior to harvesting the RNA and transferring to a nylon membrane as described above. The nylon membrane was probed with $^{32}$P-labeled CNT1 cDNA. There was no difference in the amount of CNT1 mRNA in the hSec10-overexpressing versus control cells. There was also no CNT1 mRNA seen in untransfected MDCK cells. Representative gels are shown. Quantification and statistical analysis of the gel bands were done for multiple samples using NIH Image and Microsoft Excel software and the results are shown beneath each figure.

**Figure 3:** Co-localization of GFP-hSec10p and an ER resident protein. hSec10 was cloned into the Enhanced Fluorescent Protein (EGFP, Clontech) vector, transfected into MDCK cells, and stable lines were generated. Confocal immunofluorescence microscopy was performed after staining with an antibody against the ER resident protein IP3R3. In the merged section, IP3R3 expression is shown in red and GFP-hSec10p expression in green, with areas of co-localization being yellow. Bar=10 um.

**Figure 4:** Co-immunoprecipitation (co-IP) of hSec10-myc and Sec61β. hSec10-myc overexpressing (S) and control (C) cells were grown on Transwell filters for seven days. The filter-grown cells were lysed and immunoprecipitation (IP) was performed using antibody against Sec61β (a,c) and the myc epitope tag of hSec10-myc (b,d). The IP was analyzed by SDS-PAGE followed by Western blot using antibodies against the myc epitope tag (a,b) and Sec61β (b,c). The first marker lane (M) represents an aliquot of lysate from hSec10-myc cells (no IP, Western only) and, in addition to being a marker, is used, in combination with the “self-blot” (b,c), to measure the percentage of co-immunoprecipitation between the hSec10-myc and Sec61β proteins (see text). a) hSec10-myc is shown to co-IP with Sec61β in the hSec10-myc overexpressing cells. b) Total lysate is immunoprecipitated with antibody against the myc epitope tag and then blotted for myc to determine the total amount of hSec10-myc that can be immunoprecipitated from one Transwell filter. Both lanes were from the same gel but were not contiguous. c) Total lysate is immunoprecipitated with antibody against Sec61β and then blotted for Sec61β to determine the total amount of Sec61β that can be immunoprecipitated from one Transwell filter. Equivalent amounts of Sec61β are seen, per Transwell filter, in the hSec10-myc overexpressing and control cells. d) Sec61β is shown to co-IP with hSec10-myc. e) ER proteins such as SSR and GP94 do not co-immunoprecipitate with hSec10-myc demonstrating specificity for the Sec10-myc/Sec61β interaction. M=an aliquot of cell lysate, S=hSec10-myc overexpressing cells, C=control cells.
Figure 5: Co-immunoprecipitation of endogenous exocyst complex members Sec6 and Sec8 with Sec61β. hSec10-myc overexpressing (S) and control (C) cells were grown on Transwell filters for seven days. The filter-grown cells were lysed and immunoprecipitation was performed using antibodies against Sec6p and Sec8p. The immunoprecipitate was then analyzed by SDS-PAGE followed by Western blot with antibodies against Sec61β which demonstrated an interaction between endogenous Sec6/8 and Sec61β. Furthermore, the interaction between endogenous Sec6/8 and Sec61β was greater in the cells overexpressing hSec10 compared to control cells. IP Sec6/8=immunoprecipitate with antibodies against either Sec6 or Sec8.

Figure 6: Protein secretion as determined by pulse chase and proteosome inhibition. hSec10 overexpressing (Sec10) and control cells, either treated with the proteosome inhibitor MG132 for one-hour or left untreated, were metabolically pulse-labeled for fifteen minutes with $^{35}$S-methionine and the apical and basolateral media were collected following a sixty-minute chase. Aliquots were analyzed by SDS-10% PAGE. The radioactivity associated with gp80 and other secretory proteins was unchanged in the treated versus untreated cells. Results for the apical medium are shown though similar results were also seen with the basolateral medium. Quantification of the apically secreted gp80 protein levels gave the following results: in hSec10 cells without MG132-334,097 +/- 19,381; in hSec10 cells with MG132-320,378 +/- 54,717; in control cells without MG132-148,177 +/- 21,115; and in control cells with MG132-205,143 +/- 70,248 arbitrary phosphorimager units. As previously demonstrated there was significantly more protein secreted in the hSec10 overexpressing cells compared to control cells (1). NS=not significant.
Figure 1, Lipschutz et al
Figure 2, Lipschutz et al
Figure 3, Lipschutz et al.
Figure 4, Lipschutz et al
Figure 5, Lipschutz et al.

IP Sec8  IP Sec6

KD  S  C  S  C

Blot

Sec61β
Figure 6, Lipschutz et al

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