Mammalian Vesicle Trafficking Proteins of the Endoplasmic Reticulum and Golgi Apparatus*

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Vesicle traffic propagates and maintains distinct subcellular compartments and routes secretory products from their site of synthesis to their final destinations. As a basis for the specificity of vesicular transport reactions, each step in the secretory pathway appears to be handled by a distinct set of evolutionarily conserved proteins. Mammalian proteins responsible for vesicle trafficking at early steps in the secretory pathway are not well understood. In this report, we describe rat sec22 (rsec22) and rat bet1 (rbet1), mammalian sequence homologs of yeast proteins identified as mediators of endoplasmic reticulum-to-Golgi protein transport. rsec22 and rbet1 were expressed widely in mammalian tissues, as anticipated for proteins involved in fundamental membrane trafficking reactions. Recombinant rsec22 and rbet1 proteins behaved as integral membrane components of 28 and 18 kDa, respectively, consistent with their primary structures, which contain a predicted transmembrane domain at or near the carboxyl terminus. Recombinant rsec22 and rbet1 had distinct subcellular localizations, with rsec22 residing on endoplasmic reticulum membranes and rbet1 found on Golgi membranes. Studies with brefeldin A and nocodazole indicated that rbet1 function might involve interaction with or retention in the intermediate compartment. The distinct localizations of rsec22 and rbet1 may reflect their participation in opposite directions of membrane flow between the endoplasmic reticulum and Golgi apparatus.

Vectorial membrane transport reactions are presumed to require formation of protein complexes between integral components of the transport vesicle (vesicle SNAP receptors or v-SNARES) and target membrane (target membrane SNAP receptors or t-SNARES) as well as several soluble and peripheral membrane proteins. Because distinct sets of these proteins are stationed at each membrane transport step, it has been proposed that they encode the specificity and fidelity of membrane trafficking reactions (1, 2). Despite characterization of several protein complexes involving integral proteins of the vesicle and target membranes, homologs of the yeast Sec1 protein, and rab proteins, it is not known how protein-protein interactions contribute to the specificity of targeting, docking, and fusion of transport vesicles with the target membrane.

Mechanistic insights from higher eukaryotes have been gained through the molecular characterization of nerve terminal components (3) and the study of in vitro membrane transport reconstitutions (4). Although protein-protein interactions sufficient to explain aspects of docking and fusion have been documented in the nerve terminal, where components are abundant (5, 6), the anticipated array of proteins involved at distinct transport steps has not appeared. Therefore, opportunities to evaluate the role of various protein complexes in the specificity of docking and fusion depend upon the discovery of additional vesicle trafficking proteins from distinct cellular compartments.

BET1, BOS1, and SEC22 form a set of interacting yeast genes required in endoplasmic reticulum (ER)-to-Golgi protein transport. Overexpression of BOS1 suppresses bet1 mutations, and overexpression of either BET1 or BOS1 suppresses sec22 mutations (7, 8). The accumulation of vesicles seen in sec22 mutants indicates that this mutation affects a late, post-budding step, perhaps vesicle targeting or fusion (9). Each of these genes encode small integral membrane proteins (16, 27, and 28 kDa, respectively) with hydrophilic cytoplasmically oriented N termini and central domains and C-terminal transmembrane anchors. Their small size and overall membrane topology are reminiscent of the vesicle-associated membrane protein (VAMP) family (10). Additionally, Bet1p, Bos1p, and Sec22p have all been detected as integral components of putative ER-to-Golgi transport vesicles, although it is controversial as to whether all three reside on a single vesicle class (11, 12). There appears to be a consensus at least that Bos1p and Sec22p exist on a single vesicle type, and there is recent evidence that they physically interact to activate vesicles for docking or fusion with the Golgi membrane (13). The transport vesicles also appear to contain the GTP-binding protein Ypt1p (11), whose function in ER-to-Golgi transport has been suggested to regulate the Bos1p-Sec22p interactions (13).

Yeast Sed5p (14) and its mammalian homolog syntaxin 5 (15) represent potential receptors on the Golgi membrane for targeting proteins of ER-derived transport vesicles. Recently, large protein complexes containing Sed5p, Bet1p, Bos1p, Sec22p, Sly1p, and Sec17p as well as previously uncharacterized proteins have been immunoprecipitated from detergent extracts of yeast cells arrested at a late step in ER-to-Golgi protein transport (16). Although consistent with the expectation that vesicle targeting and fusion require protein complex formation between transport step-specific proteins, these find-

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The abbreviations used are: ER, endoplasmic reticulum; VAMP, vesicle-associated membrane protein; rsec22, rat sec22; rbet1, rat bet1; EST, expressed sequence tag; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; BFA, brefeldin A; PBS, phosphate-buffered saline; β-COP, β-coatomer protein.

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ings highlighted the need to define specific roles for the unexpectedly large number of protein participants.

To further understand the mechanism of vesicle transport, we sought mammalian homologs of Sec22p and Bet1p. The presence of mammalian sequence homologs to proteins that mediate ER-to-Golgi traffic in yeast confirms the universal nature of the machinery underlying steps in the secretory pathway. As a first indication of these proteins’ function, we expressed and localized them in mammalian cells with distinctive and well-characterized morphology. Unexpectedly, the two proteins resided in membranes at opposite ends of the anticipated transport step, with rsec22 on ER membranes and rbet1 on Golgi membranes. One suggestion is that rsec22 and rbet1 are involved in the trafficking of vesicles participating in opposite directions of transport between the ER and Golgi.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning and Sequencing**—The Human Genome Sciences, Inc. data base of expressed sequence tags (ESTs) was searched and found to contain two human brain cDNA sequences (accession numbers HPD0334R (570 nucleotides) and HOSARI56R (316 nucleotides)) that encode predicted amino acid sequences 33% and 21% identical, respectively, to yeast Sec22p and Bet1p over 90- and 73-amino-acid overlap regions. Two 20-bp polymerase chain reaction (PCR) primers were designed for each sequence such that a 308-bp and a 356-bp PCR product could be generated using the human clones as a template. PCR products were generated, 32P-labeled using random hexamer labeling, and employed to screen (60°C, 6 h, × SSC) Stratagene λZAPII cDNA libraries from adult rat tissues. The human sec22 probe was used to screen 3 × 10^8 plaques from brain and 2 × 10^8 plaques from a liver cDNA library. This screening experiment resulted in finding a single positive phage from the liver library that was plaque-purified, converted to ϕBluescript KS, and designated L-sec22-1. L-sec22-1 was found to harbor a 1.4-kb cDNA insert that was restriction-mapped and sequenced using Sequenase (USB, Cleveland, OH) reaction, and 50 primers. A 5'-coding sequence of rsec22 began on nucleotide 84 of a 845-nucleotide open reading frame that was sequenced fully on both strands. The methionine designated as the first amino acid of rsec22 met the criteria for favorable translation initiation (17) and was the first methionine following a stop codon. GenBank and EMBL data base searches using the tfasta program (Genetics Computer Group, Madison, WI) revealed that rsec22 was more similar to yeast Sec22p than to any other protein or predicted protein sequence.

The human bet1 PCR probe was utilized to screen a total of 3 × 10^6 plaques from a Stratagene λZAPII adult rat brain cDNA library, resulting in a single positive clone, B-bet1-1, harboring a 1.4-kb cDNA. Sequencing revealed that this clone encoded amino acids 37–119 of a rat bet1 sequence (rbet1), but was truncated 100 nucleotides from the carboxyl terminus. A 1.4-kb PCR product from the PCR fragment was exchanged with the 1.4-kb cDNA of rsec22 and spliced into a mammalian expression construct. A rat multiple-tissue Northern blot of RNA from various tissues was purchased from CLONTECH (Palo Alto, CA). The blot was hybridized with a 32P-labeled 1.2-kb EcoRI fragment of B-bet1-1 or a 1.3-kb EcoRI fragment of L-sec22-1. Hybridizations were carried out overnight at 42°C in 5 × SSPE (0.75 M NaCl, 0.05 M NaEDTA, pH 7.4) and 50% formamide or 5% SDS at the manufacturer's instructions. Blots were washed for 40 min in 2 × SSC and 0.05% SDS at room temperature followed by 40 min in 0.1 × SSC and 0.1% SDS at 50°C.

**Expression Constructs and Transfections**—65-Nucleotide PCR primers were designed encoding the N terminus of rsec22 and rbet1 plus 10 amino acids from c-myc constituting the 9E10 epitope (18). These primers were used in conjunction with C-terminal reverse primers to amplify modified rsec22- and rbet1-encoding PCR products using L-sec22-1 or L52 as template. In the case of rsec22, a 150-bp amino-terminal Xhol restriction fragment from the PCR fragment was exchanged with the unmodified amino terminus in L-sec22-1, and the entire 1.4-kb cDNA insert was then subcloned into the pCMV mammalian expression vector (19). For rbet1, the PCR product encoding the myc amino terminus and the entire rbet1 coding sequence was directly subcloned into pCMV. To make the C-terminally deleted expression constructs, PCRs were performed on the full-length myc constructs using an upstream pcMV sense primer and antisense internal primers encoding in-frame stop codons, and the PCR products were subcloned into pCMV. All expression constructs were verified by DNA sequencing.

**COS cells** (passages 3–15) were maintained in Dulbecco's modified Eagle's medium H-16 with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate in a humidified 5% CO2 incubator. Cells (5 × 10^5) were plated on 10-cm dishes—24 h prior to transfection. For transfections, plates were rinsed twice with Dulbecco's modified Eagle's medium H-16 lacking serum and antibiotics and incubated for 40 min at 37°C with 5 ml of the same medium containing 2.5 mg of DEAE-dextran and 25 μg of pcMV construct DNA purified with QIAGEN maxi- or midiprep kits. Plates were then incubated at 37°C with 5 ml of Dulbecco's modified Eagle's medium H-16, 10% fetal bovine serum, and antibiotics plus 100 μg/ml chloroquine. After 2 h, the plates were incubated for 2.5 min at room temperature in 5 ml of Dulbecco's modified Eagle's medium H-16, fetal bovine serum, and antibiotics plus 10% dimethyl sulfoxide and then returned to 37°C in normal culture medium. The following day, culture medium was replaced, and cells were incubated for an additional 48–72 h prior to fixation. To fix cells for microscopy or harvested for extraction studies 40–48 h post-transfection.

**Drug Treatments and Immunofluorescence Microscopy**—Transfected or control COS cells (10,000) were seeded into each well of an eight-chamber microscope slide (Nunc, Naperville, IL) 24 h prior to staining. For drug treatments prior to immunofluorescence microscopy, cells were incubated for the indicated times with growth medium containing 10 μg/ml brefeldin A (BFA) (Life Technologies, Inc.; stock solution of 5 mg/ml in methanol) or 10 μM nocodazole (Sigma; stock solution of 5 mM in dimethyl sulfoxide), or both. When allowing BFA-treated cells to recover from the treatment, the BFA-containing medium was removed, and cells were washed with phosphate-buffered saline (PBS) twice and then incubated with fresh growth medium for 2–7 h prior to fixation. To fix cells for microscopy, growth medium was removed, and cells were incubated for 20 min with 2% paraformaldehyde (EM Sciences, Fort Washington, PA) in 0.1 M sodium phosphate, pH 7.0, followed by quenching twice for 10 min each with 0.1 M glycine in PBS. An alternative fix protocol involved incubation of slides in 95% ethanol at −20°C, followed by complete drying of the slides before proceeding with staining. Both fixation methods gave similar results for the experiments described.

Cell staining involved a 15-min incubation in permeabilization solution (0.4% saponin, 1% bovine serum albumin, and 2% normal goat serum in PBS) followed by incubation in permeabilization solution containing primary antibody for 1 h at room temperature. Cells were then washed three times with PBS and incubated in permeabilization solution containing affinity-purified fluorescein- or rhodamine-labeled...
goat antibodies against mouse or rabbit IgG (BioSource, International, Camarillo, CA). Slides were finally washed three times with PBS, covered with Citifluor antifouling agent (Citifluor, Canterbury, United Kingdom), and mounted beneath coverslips. Microscopy and photography were conducted using a Zeiss Axiopt microscope camera system using a 40× or 63× oil objective for a total magnification of \( \times 125 \) or \( \times 197 \) and Ektachrome 400 ASA film. Color slides were digitized and adjusted, and arranged using Adobe Photoshop software and printed using a photodigital printer.

Antibodies—Monoclonal anti-myc (9E10) ascites fluid was purchased from Berkeley Antibody Co. (Berkeley, CA) and utilized at a dilution of 1:2000. Rabbit \( \beta \)-coatomer protein (\( \beta \)-COP) antiserum raised against the EAGE peptide (20) was a gift from Dr. Suzanne Pfeffer (Stanford University) and was utilized at a dilution of 1:500. Dr. Suzanne Pfeffer also provided anti-mannose phosphate receptor antiserum (21). Rabbit calnexin antiserum was a gift from Drs. Ari Helenius and Jan Simons (Yale University) (22) and was utilized at a dilution of 1:200. A peptidylglycine \( \alpha \)-amidating monooxygenase expression construct encoding peptidylglycine \( \alpha \)-hydroxylating monooxygenase (23) and a peptidylglycine \( \alpha \)-hydroxylating monooxygenase polyclonal antibody were provided by Drs. Dick Mains and Betty Eiper (Johns Hopkins University, Baltimore). A luminal peptide-specific antibody against the intermediate compartment protein p58 was a gift from Dr. Ralf Pettersson and Ulrika Mattsson (Ludwig Institute, Stockholm, Sweden) and was useful for staining COS cells at a dilution of 1:1200. Monoclonal antiserum (clone 58K-9) against a 58-kDa Golgi peripheral protein (24) was purchased from Sigma and utilized in staining experiments at a dilution of 1:100. Rhodamine-conjugated wheat germ agglutinin was purchased from Vector Labs, Inc. (Burlingame, CA).

Rabbit syntaxin 5 antiserum was raised by injection of a bacterial glutathione S-transferase fusion protein including the syntaxin 5 cytoplasmic domain and was useful for immunostaining at a dilution of 1:1000. Four observations demonstrated that the Golgi staining obtained with this antiserum was specific for syntaxin 5. First, the localization matched that seen previously with epitope-tagged syntaxin 5 (15). Second, the antiserum recognizes recombinant syntaxin 5 overex- pression matched that seen previously with epitope-tagged syntaxin 5 (data not shown). Third, preimmune serum did not exhibit staining in COS cells (15). Fourth, an excess of glutathione S-transferase alone had no effect (data not shown). Extraction Studies—Five 10-cm culture dishes of barely confluent myc-rsec22- or myc-rbet1-transfected COS cells were harvested by trypsinization, washed once with cold PBS, and resuspended in 4 ml of ice-cold homogenization buffer (0.25 m sucrose, 20 m Hepes, pH 7.0, 2 m KCl, 1 m EDTA, 1 m dithiothreitol, 1 m phenylmethylsulfonyl fluoride, 1 m gum leupeptin, and 2 m gum apronitin). The cell sus- pension was homogenized with 25 strokes in a tight-fitting Dounce homogenizer, and unbroken cells and intact nuclei were removed by centrifugation at 1000 \( \times g \) for 15 min. In pilot Western experiments, it was established that both myc-rsec22 and myc-rbet1 could be detected solely in the insoluble material and not in 100,000 \( \times g \) cytosolic or salt extract fractions (data not shown). To construct the experiment shown in Fig. 6, one-fourth of the post-nuclear supernatant from three plates was centrifuged at 12,000 \( \times g \) for 15 min, and the resulting pellet was resuspended in homogenization buffer containing 1.5 m NaCl. This suspension was stored on ice for 30 min and then centrifuged at 100,000 \( \times g \) for 15 min, followed by adjustment of the supernatant and pellet to equal volumes of 1 \( \times SDS \) gel sample buffer. These samples are repre- sented in the first and second lanes of Fig. 6. The remaining three-fourths of the post-nuclear supernatant was adjusted to 1.5 m NaCl, stored on ice for 30 min, divided into three aliquots, and centrifuged at 12,000 \( \times g \) for 15 min. The 12,000 \( \times g \) pellets were then resuspended in homogenization buffer containing 5 m urea; 0.2 m Na2CO3, pH 11.4; or 1% Triton X-100. After 30 min on ice, each sample was centrifuged at 100,000 \( \times g \) for 15 min, and equal portions of each supernatant and pellet were adjusted to 1 \( \times SDS \) gel sample buffer. These samples are represented in the third through eighth lanes of Fig. 6. Supernatant and pellet samples were analyzed by ECL immunoblotting (Amersham Corp., Arlington Heights, IL) using 12% SDS-polyacrylamide gel electrophoresis, anti-myc monoclonal antibody 9E10, and goat anti-mouse horseradish peroxidase secondary antibodies (Zymed Laboratories, Inc., S. San Francisco, CA).
The patterns of conservation do not readily reveal that none of the stretches of three or more identical residues between rsec22 and Sec22 or rbet1 and Bet1 exhibit a 95% probability of forming a coiled-coil domain. These predictions contrast with that for rat VAMP-2, which displays a 95% probability of forming a coiled-coil domain (rsec22, 0.03% probability of forming a coiled-coil domain; Sec22, 60%; rbet1, 16%; and Bet1, 8.5%).

Hence, it is possible that other, functionally redundant isoforms substitute for rsec22 in tissues where expression is lower. Another possible explanation for the prominence of rsec22 in liver and lung is that these very active secretory tissues engage in specialized aspects of secretory actions common to all cell types. One possible explanation for the preferential expression of Sec22 is its prominent secretory role and its especially enrichment in endoplasmic reticulum compared with other cell types (27). Although no definitive structural data have been presented for Sec22, the presence of coiled-coil structures in the cytoplasmic domains of VAMP proteins suggests that coiled-coil interactions form the basis of interactions between vesicle and target membrane proteins. Although no definitive structural data have been presented for structures likely to engage in coiled-coil interactions using the COILS program (26), yeast Sec22 is the only protein among them with a substantial probability to contain a coiled-coil domain (rsec22, 0.03% probability of forming a coiled-coil domain; Sec22, 60%; rbet1, 16%; and Bet1, 8.5%).

These predictions contrast with that for rat VAMP-2, which exhibits a 95% probability of forming a coiled-coil domain. Hence, if rsec22 and rbet1 participate in the protein complexes involved in vesicle transport steps, their participation may involve interaction mechanisms other than coiled-coil binding. The significance and universality of coiled-coil mechanisms in vesicle trafficking are major issues yet to be resolved.

Subcellular Localization—Intracellular targeting of a protein to a particular organelle often provides valuable insights into its specific biological role. To examine the subcellular localization of rsec22 and rbet1, both full-length cDNAs were tested (data not shown). rbet1 appeared to be encoded by a putative transmembrane domain (amino acids 96–115) corresponding to the yeast hydrophobic stretch (amino acids 118–141), but unlike rsec22, lacks a significant C-terminal extension beyond the predicted transmembrane sequence. As with rsec22, no signal sequence was evident.

GenBank and EMBL data base searches revealed that rsec22 was more similar to yeast Sec22 than to any other protein or predicted protein sequence. rbet1 was most similar to two other mammalian vesicle trafficking proteins such as VAMP and cellubrevin, although their overall structure, size, and membrane topology are characteristic. Pairwise optimal alignments revealed that none of the stretches of three or more residues identical between rsec22 and Sec22 or rbet1 and Bet1 (see Figs. 1 and 2) were conserved between any one of these four proteins and rat or bovine VAMP/Synaptobrevin isoforms (data not shown). The patterns of conservation do not readily distinguish amino acid stretches likely to be involved in general versus transport step-specific functions.

One feature of VAMP and related proteins that may be important in protein complex formation is the predicted existence of coiled-coil structures in the cytoplasmic domains. It has been suggested that coiled-coil interactions form the basis of interactions between vesicle and target membrane proteins, although no definitive structural data have been presented (25). We analyzed the rsec22, Sec22, rbet1, and Bet1 sequences for structures likely to engage in coiled-coil interactions using the COILS program (26) and found that yeast Sec22 is the only protein among them with a substantial probability to contain a coiled-coil domain (rsec22, 0.03% probability of forming a coiled-coil domain; Sec22, 60%; rbet1, 16%; and Bet1, 8.5%).

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Tissue Expression Patterns—The tissue expression patterns of the rsec22 and rbet1 genes were examined by Northern blot analysis. As evident in Fig. 3A, which shows an autoradiogram exposure of 4 h, rsec22 was expressed as an abundant 2.6-kb transcript predominant in lung and liver. On overnight exposures, the rsec22 transcript could be detected in all the tissues tested (data not shown). rbet1 appeared to be encoded by a 1.7-kb transcript, which displayed more even expression levels across all the tissues tested (see Fig. 3B, which shows an overnight exposure), with highest expression in lung, liver, skeletal muscle, and kidney. A minor transcript of 0.76 kb was evident in liver, skeletal muscle, and testis on longer exposures. It is curious that rsec22 and rbet1 have dramatically different expression patterns despite the expectation that both proteins participate in fundamental membrane trafficking reactions common to all cell types. One possible explanation for the 4-h exposure of the rsec22 probe has a predicted size of 2.6 kb; the major species for rbet1 is 1.7 kb. The autoradiogram exposure shown for rsec22 was 4 h, while that for rbet1 was overnight. Upon overnight exposure, the rsec22 mRNA was visible in all of the lanes (not shown).
amino-terminally tagged with a c-myc epitope and transiently expressed in COS fibroblasts. myc-rsec22 and myc-rbet1 were detected by indirect immunofluorescence microscopy using the 9E10 anti-myc monoclonal antibody. As illustrated in Fig. 4 (A and C), myc-rsec22 was localized to intracellular membranes displaying a tubular/reticular pattern. This staining pattern is characteristic of the endoplasmic reticulum and overlapped significantly with that of the ER protein calnexin (Fig. 4, A versus B and C versus D) (22). The reticular ER staining for myc-rsec22 contrasted markedly with the tight perinuclear staining observed for myc-rbet1. As shown in Fig. 4 (E versus F), myc-rbet1 and the cis-Golgi protein β-COP appeared to coincide indistinguishably. The myc-rbet1 staining pattern was also indistinguishable from that generated by endogenous COS cell syntaxin 5, a proposed cis-Golgi vesicle receptor that functions in ER-to-Golgi membrane traffic (Fig. 4, I versus J) (14, 28). On the other hand, myc-rbet1 overlapped, but was more restricted than the trans-Golgi/trans-Golgi network staining generated with wheat germ agglutinin (data not shown) (29). The distinctive, nonoverlapping localizations of myc-rsec22 and myc-rbet1 suggested that the recombinant proteins were targeting correctly, as was the case for syntaxin 5 in the same system (15). The distinct localizations of the epitope-tagged proteins did not vary with expression levels in COS cells (data not shown).

Dynamics during Membrane Flow Perturbations—The dynamics of proteins in the presence of brefeldin A and other membrane flow perturbants can reveal features of their native localization and life cycle (30, 31). The microtubule-depolymerizing agent nocodazole blocks retrograde traffic between the ER and the Golgi membranes, whereas anterograde traffic, which is microtubule-independent, is not affected (32). Because it is possible that myc-rsec22 transiently visits compartments beyond the ER, we examined whether 10 μM nocodazole treatment for 1 h could trap a portion of the protein with a distinct non-ER localization. Although nocodazole caused a slight change in ER morphology, myc-rsec22 continued to colocalize extensively with the calnexin ER marker (data not shown).

Hence, epitope-tagged rsec22 did not appear to depend upon retrograde traffic for its ER localization over a 1-h time scale. Nocodazole has been shown to cause the vesiculation and dispersal of Golgi cisternae in a manner akin to the breakdown preceding mitosis (33). We examined the behavior of the Golgi vesicle trafficking proteins syntaxin 5 (endogenous) and myc-rbet1 as well as an endogenous 58-kDa Golgi protein (not to be confused with the intermediate compartment protein p58; see below) (24) after treatment with 10 μM nocodazole. After 5 or 15 min of treatment, the compact Golgi staining became more dispersed to an approximately equal degree for all three proteins, whereas by 1 h of treatment, the antigens were completely decentralized on islands randomly distributed throughout the cytoplasm. myc-rbet1 and syntaxin 5 (Fig. 5, A and B) and the 58-kDa protein and syntaxin 5 (data not shown) were found to colocalize to the same vesicles, indicating that each of these proteins resided on microtubule-sensitive Golgi cisternae.

BFA causes a block in ER-to-Golgi membrane traffic and induces Golgi proteins to return to the ER via a retrograde tubulovesicular mechanism. Since rsec22 may participate dynamically in ER-to-Golgi membrane flow, we tested whether a brefeldin A blockade of ER-to-Golgi traffic would produce an altered myc-rsec22 distribution. We found that myc-rsec22 and the ER resident protein calnexin continued to colocalize after 2 h of treatment with 10 μg/ml BFA, indicating that epitope-tagged rsec22 was not dependent on normal ER-to-Golgi traffic to maintain its characteristic ER localization (data not shown). We also examined the behavior of the Golgi vesicle trafficking proteins syntaxin 5 and myc-rbet1 as well as the 58-kDa Golgi protein upon BFA treatment. The 58-kDa protein has been previously shown to redistribute to the ER under similar conditions (34). Syntaxin 5 (Fig. 5D), like the 58-kDa protein (data not shown), took on a fine reticular pattern spreading throughout the cytoplasm and often encompassing the nuclear envelope. This fine reticular pattern is consistent with the behavior of Golgi resident proteins that redistribute to the ER (30, 32). We also observed spotty vesicular staining for syntaxin 5 (Fig. 4A) and two endogenous ER resident proteins, whereas by 1 h of treatment, the antigens were completely decentralized on islands randomly distributed throughout the cytoplasm.
and, to a lesser extent, the 58-kDa protein (data not shown), although in most cases, this did not appear markedly concentrated relative to the continuous reticular pattern. myc-rbet1 also exhibited a fine reticular pattern upon BFA treatment, indicating access to the ER; on the other hand, myc-rbet1 more often displayed a marked spotty appearance, with bright staining of cytoplasmic vesicles, fainter reticular staining, and little or no visible coverage of the nuclear envelope (Fig. 5, C and E). In BFA-treated transfected cells costained for myc-rbet1 and syntaxin 5, the spotty structures decorated by both antibodies coincided, the difference being that myc-rbet1 appeared more concentrated in the vesicular structures, whereas syntaxin 5 appeared more diluted into the fine reticular pattern encompassing the cytoplasm and often covering the nuclear envelope (Fig. 5, C versus D). The greater relative concentration of myc-rbet1 in cytoplasmic vesicles was not a transient phase during BFA treatment since it was manifest after 1, 2, 3, and 4 h of 10 μg/ml BFA treatment. It was not a distinction caused by overexpression since many cells exhibiting very weak myc-rbet1 staining levels also displayed predominantly spotty staining. We also found that endogenous syntaxin 5 and hemagglutinin-tagged syntaxin 5 (15) produced similar, mostly reticular staining patterns upon BFA treatment, whether detected with syntaxin 5 antiserum or anti-hemagglutinin monoclonal antibodies (data not shown).

Although Golgi resident proteins are primarily visible in the ER during BFA treatment, they have been shown to rapidly cycle between the ER and the intermediate compartment via a microtubule-independent anterograde pathway and a microtubule-dependent retrograde shunt mechanism (32). To deter-

Fig. 5. Double-label immunofluorescence microscopy documenting the dynamics of epitope-tagged rbet1 and endogenous syntaxin 5 upon BFA and/or nocodazole treatment of transfected COS cells. 2-h treatment with 10 μM nocodazole: A, myc-rbet1 stained with anti-myc monoclonal antibodies; B, syntaxin 5 stained with anti-syntaxin 5 antiserum. 2-h treatment with 10 μg/ml BFA: C and E, myc-rbet1; D, syntaxin 5; F, p58, an intermediate compartment protein, stained with anti-p58 antiserum. 2-h treatment with 10 μg/ml BFA followed by 1-h treatment with 10 μg/ml BFA plus 10 μM nocodazole: G, myc-rbet1; H, syntaxin 5. 1-h treatment with 10 μg/ml BFA followed by 4 h of recovery in normal growth medium: I, myc-rbet1; J, syntaxin 5. Bar = 15 μm (A–F, I, and J) and 34 μm (G and H).
mine if the spotty structures visible during BFA treatment represented intermediate compartment vesicles, we costained myc-rbet1-transfected cells for myc-rbet1 and p58, a protein that persists in vesicular intermediate compartment elements during BFA treatment (35). As seen in Fig. 5 (E and F), the vesicles containing myc-rbet1 extensively overlap with the structures containing p58, suggesting that myc-rbet1 has access to the intermediate compartment during BFA treatment. To determine if myc-rbet1 and syntaxin 5 cycle between the ER and the intermediate compartment in the presence of BFA, we treated cells for 2 h with BFA followed by 1 h with BFA plus 10 μM nocodazole to block retrograde movement to the ER. This treatment causes Golgi proteins participating in BFA-induced ER-intermediate compartment cycling to accumulate in the intermediate compartment (32). As shown in Fig. 5 (G and H), this treatment markedly intensified the coincident spotty vesicular staining of myc-rbet1 and syntaxin 5, indicating that both proteins visited intermediate compartment vesicles transiently during BFA treatment. We found a similar intensification of colocalizing spotty staining for the 58-kDa protein as well (data not shown).

Our results indicate that the Golgi vesicle trafficking proteins myc-rbet1 and syntaxin 5 follow the pathway taken by the 58-kDa Golgi protein and several other Golgi resident proteins (30) during BFA treatment. This pathway includes access to the ER as well as to cytoplasmic intermediate compartment vesicles. This is in contrast to proteins that persist stably in the intermediate compartment (31, 35), fuse with endosomal compartments (36, 37), or become cytosolic (34) in response to BFA. The apparent tendency of myc-rbet1 to concentrate in intermediate compartment vesicles during BFA treatment could indicate participation in an intermediate compartment retention mechanism. Perhaps normal rbet1 function includes interaction with this compartment. On the other hand, a slower depletion from the Golgi membrane in response to BFA, via intermediate compartment vesicles, could account for the marked staining of cytoplasmic vesicles. Such a sluggish retrograde movement from the Golgi could indicate participation in Golgi retention mechanisms.

The kinetics of BFA effects on myc-rbet1 and syntaxin 5 differed. Whereas the tight perinuclear syntaxin 5 staining was well dispersed within 5 min of BFA addition, the tight perinuclear myc-rbet1 staining was often not well dispersed until 1 h after BFA addition. BFA disruption of both proteins was fully reversible, with return of a compact Golgi localization over 2–7 h following a 1-h BFA exposure (Fig. 5, I and J). Despite the different rates observed for initial disruption and the difference in the concentration in intermediate compartment elements, myc-rbet1 and syntaxin 5 staining returned in parallel to their perinuclear location following removal of BFA. In no case did syntaxin 5 or myc-rbet1 appear to reassemble into a compact Golgi structure while the other protein remained in dispersed elements. Hence, different mechanisms limit the rate at which these proteins initially traverse the retrograde pathway out of the Golgi membrane and finally return via anterograde movement to the newly formed Golgi membrane. We observed that the reassembly of compact perinuclear staining was retarded in myc-rbet1-transfected cells relative to nontransfected COS cells or myc-rsec22- or myc-rbet11TM-transfected (see below) cells (data not shown). After 4 h of recovery from BFA, a compact perinuclear syntaxin 5 staining was evident in 22% of cells transfected with myc-rbet1, 42% of cells transfected with myc-rsec22 or myc-rbet11TM, and 37% of nontransfected COS cells. By 7 h post-BFA treatment, ~47% of cells exhibited a compact perinuclear distribution of syntaxin 5, regardless of their transfection status. 50% is approximately the fraction exhibiting this morphology in untreated cells, the remainder being aberrations due to multinucleated or otherwise misshapen cells or poor staining. Although this effect of the myc-rbet1 protein is not uniquely interpretable in terms of rbet1 function, it may indicate that rbet1 can mediate the delicate balance of membrane flow into and out of the Golgi, consistent with its potential role as a vesicle trafficking protein for this dynamic organelle.

Membrane Association—rsec22 and rbet1 are predicted to be integral membrane proteins. In support of their integral membrane nature, transiently expressed myc-rsec22 and myc-rbet1 were detected using immunoblotting in COS cell extracts and found to reside in particulate matter partially pelleted at 12,000 × g and fully sedimentable at 100,000 × g (data not shown). As shown in Fig. 6, the apparent association with membranes resisted disruption by high salt, urea, and high pH, but both proteins could be substantially solubilized with nonionic detergent. These properties are highly consistent with a direct association of rsec22 and rbet1 with the hydrophobic core of a lipid bilayer. It is noteworthy that a significant fraction of myc-rbet1 was not extracted with Triton X-100. A similar behavior has been observed for several resident Golgi and intermediate compartment proteins. This property has been attributed to protein oligomerization and may be an important mode of retention within a specific organelle subcompartment or membrane domain (38, 39).

For both myc-rsec22 and myc-rbet1, only a single, transfection-specific immunoreactive protein band was observed (data not shown). myc-rsec22 had an SDS gel mobility of 28 kDa, closely matching its predicted molecular mass. The myc-rbet1 band appeared larger than predicted from its sequence (18 versus 13.2 kDa for rbet1 and 1.1 kDa for the myc tag), a feature shared with VAMP and which could indicate the presence of post-translational modifications or SDS-resistant secondary structures.

Yeast Sec22p has been observed to associate tightly with another potential vesicle targeting protein, Bos1p, on ER-to-Golgi transport vesicles (13). A propensity to undergo protein-protein interactions could contribute to the localization of these trafficking proteins at their proper membrane station. This notion led us to express and localize by immunofluorescence in COS cells myc-rsec22 and myc-rbet1 proteins with C-terminal deletions removing the putative membrane anchors. myc-rsec22 lacking a putative transmembrane domain and luminal sequence (designated myc-rsec22TM) appeared diffuse throughout the cell and no longer visibly associated with ER

![Fig. 6. Epitope-tagged rsec22 (A) and rbet1 (B) behave as integral membrane proteins in transfected COS cells. Post-nuclear membrane pellet fractions were extracted with various disruptive agents and centrifuged at 100,000 × g, and the resulting supernatant (S) and pellet (P) were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-myc monoclonal antibodies. The membranes in the first and second lanes were extracted with 1.5 M NaCl at pH 7.0. For the third through eighth lanes, membranes were first extracted with 1.5 M NaCl and then with 5 M urea at pH 7.0 (third and fourth lanes), 0.2 M sodium carbonate at pH 11.4 (fifth and sixth lanes), or 1% Triton X-100 at pH 7.0 (seventh and eighth lanes). myc-rsec22 migrated as a 28-kDa protein, and myc-rbet1 as an 18-kDa protein, myc-rsec22 and myc-rbet1 could be detected only in membrane fractions and only in transfected COS cells (not shown).](image)
tubules (data not shown). This indicated a requirement for the rsec22 transmembrane and/or luminal domain for membrane attachment and association with ER elements. myc-rbet1 lacking its putative transmembrane sequence (myc-rbet1ΔTM) also exhibited a diffuse staining pattern (data not shown), indicating that the 23-amino acid putative transmembrane sequence was required for both membrane association and Golgi localization of myc-rbet1. Since it was possible that the deleted proteins could have dominant effects on the secretory pathway in COS cells, we examined the morphology and staining pattern of several secretory organelles in cells expressing myc-rsec22ΔTM and myc-rbet1ΔTM. We found no visible change in ER membranes as marked by calnexin, in the intermediate compartment as marked by p58, in the Golgi membrane as marked by β-COP and syntaxin 5, in the trans-Golgi/trans-Golgi network as marked by wheat germ agglutinin, in the trans-Golgi network/endoosomes as marked by the mannose phosphate receptor, or in the apparent distribution of a constitutively secreted cargo protein, peptidylglycine α-amidating monooxygenase (see "Experimental Procedures"). cotransfected with the epitope-tagged proteins. These data suggest that the deleted constructs did not exert a dominant effect on the secretory pathway, an outcome possibly explained by their inability to interact with the proper membrane compartment. Note that relatively small perturbations in vesicle trafficking might not have been detectable using these morphological criteria.

**DISCUSSION**

**Mammalian Sec22 and Bet1 Homologs—**The simplest interpretation of our data is that rsec22 and rbet1 represent mammalian homologs of the yeast ER-to-Golgi vesicle trafficking proteins Sec22 and Bet1. This interpretation is based upon the relatively high sequence identity despite the vast evolutionary distance between yeast and mammalian cells. rsec22 and rbet1 exhibit identities of 32 and 21% to their potential yeast counterparts, respectively. As a basis for comparison, rat n-sec1 and yeast Sec1p share 28% identity, and syntaxin 5 and Sed5p share 35% identity. The sequence similarity between rsec22 and Sec22p is particularly convincing since identities are observed throughout the length of sequence overlap. Although the identity between rbet1 and Bet1p is lower and concentrated primarily around rbet1 amino acid 48, the existence of a C-terminal transmembrane domain at precisely the homologous position demonstrates that the proteins are related. A second argument for a homologous relationship involves the subcellular localizations of epitope-tagged rsec22 and rbet1. These recombinant type II membrane proteins both distinctly localize to compartments within the anticipated transport step, with myc-rsec22 localizing on the ER and myc-rbet1 on membranes indistinguishable from the cis-Golgi. These arguments make it likely that rsec22 and rbet1 play functional roles in ER-to-Golgi transport that are conserved from yeast to mammals.

Despite these similarities, we cannot rule out the possibility that rsec22 and/or rbet1 participates in steps other than ER-to-Golgi transport. rsec22 or rbet1 may represent a vesicle transport protein involved at distinct transport steps for which yeast proteins have not yet been recognized or correctly assigned. As an example, rbet1 could mediate intra-Golgi rather than ER-to-Golgi transport. Perhaps the present rbet1 is one of several Bet1-like proteins that mammalian cells have evolved for distinct transport steps. It is not yet known how many homologs of yeast Sec22p and Bet1p exist in mammalian species, or if the two proteins characterized here are the most similar homologs.

**Localizations and Dynamics—**We found that epitope-tagged rsec22 and rbet1 exhibited membrane localizations apparently limited to the ER and the Golgi membranes, respectively. Although in some of the transfected cells recombinant rsec22 and rbet1 may have been expressed at higher levels than those that occur endogenously in fibroblasts, we observed no difference in the localizations between high and low expressing COS cells (data not shown). Furthermore, the distinctive, nonoverlapping localizations suggest that the epitope-tagged proteins were targeting correctly, as was the case for epitope-tagged syntaxin 5 expressed in the same system (15).

Although both rsec22 and rbet1 localized to compartments within the anticipated transport step, it is noteworthy that they were observed only on the end point compartments (ER and Golgi), rather than on transport vesicles or other intermediates. Since yeast Sec22p and Bet1p have been biochemically detected on putative ER-to-Golgi transport vesicles, this was somewhat surprising. This situation differs from that of the mammalian vesicle transport protein VAMP/synaptobrevin, where localization to vesicles is manifest (40). Unlike synaptic vesicles, ER-to-Golgi transport vesicles are not anticipated to accumulate under normal conditions and hence might not be evident by fluorescence microscopy. Since vesicular transport between the ER and the Golgi membrane must be rapid and continuous, the total pool of each required vesicle protein should be substantially larger than that pool present on transport vesicles at any given time, with the resting pool accumulating on the donor, the acceptor, or both end compartments. It thus seems possible that rsec22 and/or rbet1 represents a transport vesicle component, as predicted based on their yeast sequence homologs, but are only evident at one end point of the transport cycle. A simple explanation for their opposing localizations would be that rsec22 and rbet1 may be involved in opposite directions of transport between ER and Golgi membranes. However, the possibility that rsec22 and/or rbet1 does not represent a transport vesicle component should also be considered since their localizations are also compatible with ER or Golgi resident trafficking roles.

The behavior of Golgi vesicle trafficking proteins in the presence of BFA had not been previously reported. We found that BFA induced myc-rbet1 to enter an ER-intermediate compartment cycling pathway like syntaxin 5 and most previously characterized Golgi proteins. However, myc-rbet1 responded more slowly to BFA and accumulated more profoundly in intermediate compartment vesicles. This behavior may indicate that rbet1 participates in Golgi and/or intermediate compartment retention mechanisms, possibly involving oligomerization, but does not uniquely favor a resident versus a vesicular function. The discovery of mammalian trafficking proteins that localize to the ER and Golgi apparatus provides valuable agents for mechanistic studies that will undoubtedly entail in vitro transport assays (41), providing functional correlates for protein-protein interactions involving a growing collection of trafficking proteins.

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