Sec15 Is an Effector for the Rab11 GTPase in Mammalian Cells*

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Rab/Ypt GTPases play key roles in the regulation of vesicular trafficking. They perform most of their functions in a GTP-bound form by interacting with specific downstream effectors. The exocyst is a complex of eight polypeptides involved in constitutive secretion and functions as an effector for multiple Ras-related small GTPases, including the Rab protein Sec4p in yeast. In this study, we have examined the localization and function of the Sec15 exocyst subunit in mammalian cells. Overexpressed Sec15 associated with clusters of tubular-vesicular elements that were concentrated in the perinuclear region. The tubular-vesicular clusters were dispersed throughout the cytoplasm upon treatment with the microtubule-depolymerizing agent nocodazole and were accessible to endocytosed transferrin, but not exocytic cargo (vesicular stomatitis virus glycoprotein). Consistent with these observations, Sec15 colocalized selectively with the recycling endosome marker Rab11 and exhibited a GTP-dependent interaction with the Rab11 GTPase, but not with Rab4, Rab6, or Rab7. These findings provide the first evidence that the exocyst functions as a Rab effector complex in mammalian cells.

Rab/Ypt proteins are members of the Ras superfamily of small GTP-binding proteins that play key roles in the regulation of intracellular vesicular trafficking (1, 2). The Rab proteins encompass a large family of related GTPases that function predominantly in distinct trafficking pathways. The Rab functional cycle is coupled to GTP binding and hydrolysis, which are catalyzed by guanine nucleotide exchange factors and GTPase-activating proteins, respectively. In the GTP-bound state, Rab proteins are recruited to membranes where they associate with specific downstream effector molecules that direct vesicle targeting and docking to the appropriate acceptor compartment. It is emerging that GTP-bound Rab proteins regulate a diverse array of effector molecules, including factors that promote vesicle formation, motility, docking, and fusion (3). GTPase-activating protein-catalyzed GTP hydrolysis results in the dissociation of effector complexes and extraction of GDP-bound Rab from membranes.

Rab11 is a ubiquitously expressed Rab protein that is involved in the endosomal recycling pathway in mammalian cells. It colocalizes with the transferrin receptor (TfnR) on pericentriolar recycling endosomes (REs) and is involved in recycling of transferrin (Tfn) to the plasma membrane (4, 5). Rab11 has also been implicated in apical recycling and transcytosis in Madin-Darby canine kidney cells (6) and trans-Golgi network (TGN) to plasma membrane trafficking via REs in baby hamster kidney cells (7). Several Rab11 effectors have been described that are involved in recycling, including Rab11-binding protein/rabphilin-11, pp75/Rip11, myosin Vb, Rab11-FIP1, Rab11-FIP4/Eferin, Rab11-FIP4, and Rab-coupling protein (8, 9).

The exocyst complex (also known as the Sec6/8 complex), one of the more extensively studied Rab effectors, is composed of eight subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) in Saccharomyces cerevisiae and mammalian cells (10, 11). It is required for constitutive secretion as well as polarized exocytosis (12). In yeast, the Sec15p subunit interacts specifically with GTP-bound Sec4p (13), a Rab protein involved in secretion. The association of activated Sec4p with Sec15p on secretory vesicles is believed to regulate interactions between exocyst subunits, leading to vesicle docking with the plasma membrane at sites defined by Sec3p (13). The molecular basis for exocyst function in vesicle targeting in higher organisms has remained obscure.

EXPERIMENTAL PROCEDURES

Reagents—An anti-Sec15 polyclonal antibody was prepared by immunization of rabbits with a C-terminal peptide (KDTSKKKNIFAFQRKNDRDRQKC) conjugated to diphtheria toxin. The antibody was affinity-purified from the antiserum using the same peptide immobilized on Amino Link Plus coupling gel ( Pierce). Mouse anti-Xpress monoclonal antibody and LipofectAMINE transfection reagent were purchased from Invitrogen. Rabbit anti-glutathione S-transferase (GST) and anti-Rab11 polyclonal antibodies were from Zymed Laboratories Inc. (South San Francisco, CA). Anti-green fluorescent protein (GFP) monoclonal antibody, Alexa Fluor-conjugated secondary antibodies, and Texas Red-labeled Tfn were purchased from Molecular Probes, Inc. (Eugene, OR). Anti-Sec6 monoclonal antibody (clone 9H5) was from Stressgen Biotech Corp. (Victoria, Canada). All other reagents were from Sigma unless indicated otherwise.

Construction of Expression Plasmids—The clones encoding Sec10 and Sec15 were obtained from Dr. R. H. Scheller (Stanford University, Stanford, CA), and human TfnR (hTfnR) was from Drs. M. Silverman and G. Banker (Oregon Health Sciences University, Portland, OR). Complementary DNAs encoding wild-type Sec15 (amino acids 2–822), Sec10 (amino acids 2–707), and TfnR were amplified by PCR and subcloned into the pcDNA3-Shuttle vector (Invitrogen). The cDNA clones encoding wild-type Rab4a, Rab6a, Rab7, and Rab11 were generated by PCR from a 3T3-L1 adipocyte cDNA library and subcloned into the pPCR-Script-amp shuttle vector (Stratagene, La Jolla, CA). The Rab11 mutants Rab11S252N and Rab11Q70L were generated using the QuikChange site-directed mutagenesis kit (Stratagene) ac-

tathione S-transferase; GFP, green fluorescent protein; hTfnR, human transferrin receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; GTP-γ-S, guanosine 5’-O-(3-thiotriphosphate); GDPβS, guanosine 5’-O-(2-thio-
diphosphate); HRP, horseradish peroxidase; VSVG, vesicular stomatitis virus glycoprotein.

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cording to the manufacturer’s instructions. For construction of expression vectors, the cDNAs were inserted in-frame into the following expression vectors: pCDNA3-HisMax-C (Invitrogen) for Xpress-tagged proteins, pEGFP-C (Clontech) for GFP fusion proteins, pEUB (14) for mammalian expression of GST fusion proteins, and pET28a (Novagen, Madison, WI) for bacterial expression of histidine-tagged Rab11 (His6-Rab11).

Cell Culture, Transient Transfections, and Fluorescence Microscopy—CHO, Chinese hamster ovary (CHO), and 293 cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (CIS Biosciences, Parkville, Victoria, Australia) and 100 units/ml penicillin and streptomycin. Transfection of CHO cells was performed using the LipofectAMINE reagent according to the manufacturer’s instructions. For COS-7 and 293 cells, transfections were performed by electroporation. A 0.2-ml aliquot of cells in suspension was added to a 50-ml mixture containing plasmid DNA (15 μg each plasmid) in 0.15 M NaCl and electroporated at 200 V and 975 microfarads. For microscopy experiments, the cells were plated on glass coverslips and allowed to recover for 16–20 h at 37 °C. The transfected cells were washed three times with phosphate-buffered saline (PBS), fixed with 3% glutaraldehyde in 0.08 M sodium cacodylate buffer for 1 h at 4 °C, and embedded in 1% (w/v) sucrose, scraped from the dishes, and pelleted by centrifugation. Ultrathin cryosections were prepared using a modified Tokuyasu technique (17). Immunolabeling of the cryosections was performed using a mouse anti-GFP monoclonal antibody (4 g/ml; Molecular Probes, Inc.) or an equivalent concentration of a mouse anti-Aspergillus niger IgG1 isotype control monoclonal antibody (Dako Corp.), followed by 10-nm gold-conjugated goat anti-mouse IgG at a dilution of 1:20.

RESULTS

Localization of Overexpressed Sec15—To investigate the subcellular localization of Sec15, we initially generated an affinity-purified polyclonal antibody against a Sec15 C-terminal peptide. In immunofluorescence microscopy experiments, relatively high concentrations of the antibody were required to detect a signal above background, and in undifferentiated PC12 cells, the staining was distributed uniformly throughout the cytoplasm with no evidence of the previously reported perinuclear enrichment (18). Based on these data, it was concluded that the anti-Sec15 peptide antibody is not suitable for immunofluorescence studies. As an alternative approach, we used confocal fluorescence microscopy to examine the localization of overexpressed Sec15 with an N-terminal Xpress epitope tag (Xpress-Sec15) or a GFP-Sec15 fusion protein in transfected cells (Fig. 1). Initial immunoblot experiments confirmed that the expression vectors directed the synthesis of Sec15 polypeptides of the appropriate molecular mass (data not shown).

Strikingly, overexpression of either GFP-Sec15 or Xpress-Sec15 in COS-7 cells resulted in the formation of numerous intense dots of fluorescence that were concentrated in the perinuclear region (Fig. 1, A and B). In addition, diffuse intracellular fluorescence was apparent, consistent with the existence of a cytosolic pool of the overexpressed protein. This pattern of fluorescence was observed upon transfection for time periods of 4 h up to at least 48 h. A similar localization was observed in transiently transfected CHO and 293 cells, where discrete dots of GFP-Sec15 fluorescence were detected in the perinuclear region (Fig. 1, C and D). The dots of fluorescence were not observed in cells transfected in parallel with a GFP vector control. It should be emphasized that the observed localization of Sec15 is likely to result from overexpression and may not reflect its subcellular distribution at normal expression levels. Interestingly, endogenous Sec15 has recently been demonstrated to localize to the perinuclear region in undifferentiated PC12 cells and to redistribute to the growing neurite and growth cone upon differentiation (18).

Yeast Two-hybrid Interaction Assays—The two-hybrid assay was performed as described previously (15). The cDNAs encoding Sec15 and the Rab proteins were ligated into the vectors pEG202 (bait) and pJG4-5 (prey), respectively. Yeast strain EGY48 cells were cotransformed with the plasmids and plated onto synthetic medium lacking uracil, tryptophan, and histidine. The transformed cells were subsequently plated onto medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as substrate and galactose to induce expression of the prey fusion protein. The binding affinities were quantitated in triplicate using a β-galactosidase assay.

Electron Microscopy—CHO cells were cotransfected with expression vectors encoding GFP-Sec15 and Xpress-hTfnR. Twenty hours after transfection, cells were incubated with serum-free medium for an additional 2 h at 37 °C. Subsequently, the medium was supplemented with 25 μg/ml human Tfn conjugated to HRP (Tfn-HRP; Rockland Inc., Gilbertsville, PA) for 30 min at 37 °C. The 3,3′-diaminobenzidine reaction was performed as described previously (16). Following the 3,3′-diaminobenzidine reaction, the cells were placed on ice, washed three times with PBS, and fixed using 2% paraformaldehyde and 0.05% glutaraldehyde in 0.08 M Sorensen’s buffer for 1 h at 0 °C. The fixed cells were washed twice with 0.08 M Sorensen’s buffer supplemented with 5% (w/v) sucrose, scraped from the dishes, and pelletted by centrifugation. Ultrathin cryosections were prepared using a modified Tokuyasu technique (17). Immunolabeling of the cryosections was performed using a mouse anti-GFP monoclonal antibody (4 μg/ml; Molecular Probes, Inc.) or an equivalent concentration of a mouse anti-Aspergillus niger IgG1 isotype control monoclonal antibody (Dako Corp.), followed by 10-nm gold-conjugated goat anti-mouse IgG at a dilution of 1:20.
Sec15 Is a Rab11 Effector

Overexpressed Sec15 associates with punctate perinuclear structures in mammalian cells. The localization of Sec15 was examined in COS-7 cells transiently transfected with expression vectors encoding GFP-Sec15 (A) or Xpress-Sec15 (B) and in CHO (C) or 293 (D) cells expressing GFP-Sec15. The cells were fixed and permeabilized 20 h after transfection and examined by confocal fluorescence microscopy. Single optical sections of the transfected cells are shown.

Whereas in control cells treated with vehicle (0.3% Me2SO), their perinuclear localization was maintained (Fig. 2). These findings suggested that the observed localization of Sec15 resulted from its association with a microtubule-tethered compartment(s) or vesicle cluster and not from nonspecific aggregation of the overexpressed protein. The observation that the dots appeared unaltered despite their redistribution within the cytoplasm also suggested that their formation did not result from a direct association of Sec15 with microtubules.

Recent studies have revealed that Sec10 associates directly with Sec15 in yeast and mammalian cells (13, 19). Therefore, we examined whether Sec10 was recruited to the fluorescent dots formed upon overexpression of Sec15 (Fig. 3). In this experiment, COS-7 cells were transfected with expression vectors encoding Xpress-Sec10 alone (Fig. 3A) or in combination with GFP-Sec15 (Fig. 3B). When expressed alone, Xpress-Sec10 exhibited diffuse cytosolic staining, consistent with previous observations (19). In contrast, when coexpressed with GFP-Sec15, Xpress-Sec10 exhibited a dramatic redistribution from the cytosol to the Sec15-positive structures. This observation suggests that GFP-Sec15 retains the ability to interact with Sec10 when overexpressed in mammalian cells and is therefore a functional molecule. In contrast, GFP-Sec15 did not exhibit detectable overlap with Sec6 (Fig. 3C), raising the possibility that Sec10 may be recruited to the Sec15-positive structures independently of other exocyst subunits.

Since exocyst subunits have been implicated in the TGN to plasma membrane limb of the secretory pathway, it was important to establish whether the perinuclear dots of fluorescence observed upon overexpression of Sec15 represent elements of the TGN or TGN-derived vesicles. To test this, the ts045 temperature-sensitive mutant of vesicular stomatitis virus glycoprotein (VSVG) fused to GFP (20) was utilized as an exocytic reporter. ts045-VSVG is reversibly misfolded and retained in the endoplasmic reticulum at the restrictive temperature (39.5 °C) and is able to exit the endoplasmic reticulum and traffic to the plasma membrane following incubation at the permissive temperature (32 °C). However, if the cells are shifted directly from the restrictive temperature to 19 °C, ts045-VSVG is transported from the endoplasmic reticulum and subsequently arrested in Golgi/TGN compartments. Therefore, cells expressing ts045-VSVG-GFP and Xpress-Sec15 were incubated at 39.5 °C for 20 h and then at 19 °C for 2 h to trap ts045-VSVG-GFP in the Golgi/TGN and examined by microscopy (Fig. 4). The ts045-VSVG-GFP fusion protein used for these studies has been shown previously to exhibit exocytic trafficking properties that are indistinguishable from those of untagged VSVG in COS cells (20).

Following temperature shift from 39.5 to 19 °C for 2 h, a patch of ts045-VSVG-GFP fluorescence was detected in COS-7 cells, characteristic of the Golgi (Fig. 4A). As expected, Xpress-Sec15 labeled punctate structures that were concentrated in the perinuclear region in cotransfected cells (Fig. 4B). Merged images (Fig. 4, C and D) revealed that there was minimal overlap between the staining patterns of ts045-VSVG-GFP and Xpress-Sec15 under these conditions. The limited overlap that was observed was most likely a consequence of close proximity of Sec15 and ts045-VSVG in the perinuclear region rather than co-association on Golgi/TGN membranes since the structures labeled by Xpress-Sec15 were clearly distinct from the single patch of fluorescence defined by ts045-VSVG. In light of previous observations concerning the localization of overexpressed Sec15p in yeast (21), we considered it possible that the Sec15-positive structures might represent a cluster of accumulated exocytic vesicles. To test this, transfected cells that had been held at 19 °C for 2 h were subsequently shifted to 32 °C to allow the formation of TGN-derived vesicular intermediates and transport of ts045-VSVG-GFP to the plasma membrane. We were unable to detect significant colocalization between ts045-VSVG-GFP and Xpress-Sec15 following temperature shift to 32 °C for 30 min (Fig. 4, E–H). At late time points, a rim of ts045-VSVG-GFP fluorescence around the cell surface was observed in Sec15-expressing cells, consistent with progression to the plasma membrane (data not shown).

The lack of colocalization between ts045-VSVG-GFP and overexpressed Sec15 prompted us to examine whether the structures labeled by the exocyst subunit are accessible to endocytic cargo. The Tfn recycling pathway has been studied extensively in mammalian cells. Following its binding to TfnR at the cell surface, Tfn is internalized and transported through early endosomes, from where it is recycled back to the plasma membrane via the RE compartment. To investigate whether the Sec15-positive structures are accessible to internalized Tfn, we transiently transfected COS-7 cells with expression vectors encoding GFP-Sec15 and hTfnR. Previous studies have established that recombinant hTfnR functions in a manner that is indistinguishable from the endogenous receptor in transfected cells (22). A pulse-chase experiment was performed in which...
the cotransfected cells were incubated in the presence of biotinylated Tfn for 5 min at 37 °C (pulse) and then chased with unlabeled Tfn for increasing times. The localization of GFP-Sec15 and endocytosed biotinylated Tfn was examined by microscopy (Fig. 5). When trafficking was terminated immediately following the pulse (0 min), the endocytosed Tfn labeled dispersed punctate structures that were characteristic of early endosomes, and there was little colocalization with GFP-Sec15. The rapid internalization of Tfn suggests that overexpression of Sec15 does not inhibit endocytosis. Interestingly, at the 10-min chase time, we observed partial overlap of internalized Tfn with GFP-Sec15 in the perinuclear region, and at later time points (20 and 30 min), substantial overlap was observed. Indeed, after a chase time of 30 min, the punctate perinuclear structures that were labeled for Sec15 were also predominantly Tfn-positive. These observations indicate that overexpressed Sec15 is associated with an endosomal compartment that is accessible to internalized Tfn.

**Overexpressed Sec15 Associates with Elements of the Recycling Endosome System**—The kinetics of the appearance of internalized Tfn in the Sec15-positive structures (Fig. 5) are consistent with the possibility that the exocyst subunit associates with elements of the RE system (5, 23). In light of this, we performed confocal fluorescence microscopy to confirm the lack of colocalization between GFP-Sec15 and the endosome markers EEA1 (early endosomes) and LAMP2 (late endosomes/lysosomes). We found that although the compartments labeled by endogenous EEA1 or LAMP2 were in close juxtaposition to the dots of fluorescence observed for Sec15, their localizations were predominantly distinct from the exocyst subunit (Fig. 6). The lack of colocalization with EEA1 is consistent with the observation that Sec15 did not overlap with the punctate Tfn-positive structures that were detected at early time points of internalization (0-min chase) (Fig. 5) and indicates that it does not label early endosomes.

To confirm the colocalization between overexpressed Sec15 and internalized Tfn, confocal fluorescence microscopy was performed. CHO cells were transiently transfected with expression vectors encoding GFP-Sec15 and hTfnR. Twenty hours after transfection, the cells were incubated with Tfn-HRP for 30 min at 37 °C, and endosomal compartments were labeled
with electron-dense peroxidase-3,3′-diaminobenzidine reaction product (16). Following fixation, ultrathin cryosections prepared from the transfected cells were incubated with an anti-GFP monoclonal antibody, followed by a 10-nm gold particle-conjugated anti-mouse IgG secondary reagent (Fig. 7). We observed that GFP-Sec15 consistently labeled clusters of 50–75-nm diameter tubular/vesicular profiles that were Tfn-HRP-positive (Fig. 7, A and B). Labeling for the exocyst subunit was essentially restricted to these structures with the exception of a relatively low level of cytoplasmic staining. The tubular/vesicular clusters did not appear to be surrounded by a membrane, indicating that they are distinct from multivesicular bodies/late endosomal compartments. Gold particles were not detected on the Tfn-HRP-positive tubular/vesicular clusters when the anti-GFP antibody was substituted for an isotype control (Fig. 7C), indicating that the detection of GFP-Sec15 was specific.

The strong colocalization observed between Sec15 and internalized Tfn prompted us to examine whether overexpression of the exocyst subunit might inhibit endosomal recycling. We were unable to perform a biochemical recycling assay due to low Sec15 transfection efficiencies. Therefore, a pulse-chase experiment was performed in which COS-7 cells expressing GFP-Sec15 (or control GFP) and hTfnR were incubated with Texas Red-labeled Tfn for 5 min at 37 °C (pulse) and chased with excess holo-Tfn for 40 min at 37 °C. Retention of internalized Tfn in transfected cells was evaluated by fluorescence microscopy (Fig. 8). When trafficking was terminated immediately following the pulse, Tfn was distributed throughout the cytoplasm in control GFP-expressing cells, reflecting transport to early endosomes. In cells expressing GFP-Sec15, the internalized Tfn exhibited a similar dispersed localization, although in this case, there was a higher level of perinuclear staining, which is likely to have resulted from arrival of a pool of the internalized protein in the Sec15-positive tubular/vesicular clusters. After the 40-min chase, the intracellular Tfn staining was diminished significantly due to endocytic recycling and release from the control GFP-transfected cells. In contrast, in cells expressing GFP-Sec15, the internalized Tfn colocalized precisely with the exocyst subunit and exhibited a significantly higher level of intracellular staining than observed in the control cells at the same chase time. These observations suggest that internalized Tfn accumulates in the Sec15-positive structures, consistent with a possible inhibitory effect on recycling through the RE system.

**Sec15 Is an Effector Protein for Rab11**—To investigate further the association of overexpressed Sec15 with the RE system, we examined its degree of colocalization with a series of well characterized Rab proteins (Fig. 9). For this experiment, COS-7 cells were cotransfected with expression vectors encoding GFP-Sec15 and Xpress-tagged Rab4a, Rab6a, Rab7, or Rab11a, markers for early endosomes (24), the Golgi compartment (25), late endosomes/lysosomes (26), and REs (5), respectively. Although some overlap between Rab4a, Rab6a, or Rab7 and Sec15 was apparent, it was clear that their localizations

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**Fig. 6.** Sec15 does not associate with endocytic compartments defined by EEA1 or LAMP2. COS-7 cells were transiently transfected with an expression vector encoding GFP-Sec15 and immunostained for endogenous EEA1 or LAMP2 as indicated. Single plane confocal microscope images are shown.

**Fig. 7.** Colocalization between Sec15 and internalized Tfn at the ultrastructural level. CHO cells were transiently transfected with expression vectors encoding GFP-Sec15 and hTfnR. Twenty hours after transfection, the cells were incubated with Tfn-HRP for 30 min at 37 °C and subjected to 3,3′-diaminobenzidine cytochemistry. Ultrathin cryosections were incubated with an anti-GFP monoclonal antibody (A) or an isotype control (C), followed by a 10-nm gold particle-conjugated anti-mouse IgG secondary antibody. B is a higher magnification image of the Sec15/Tfn-HRP-positive region shown in A. Localization of GFP-Sec15 to Tfn-positive tubular/vesicular clusters was apparent in the cytoplasm of transfected cells. Scale bars = 200 nm.
were predominantly distinct (Fig. 9). In contrast, Rab11a exhibited a striking colocalization with the GFP-Sec15-positive tubular/vesicular clusters. The localization data are reminiscent of studies in yeast in which Sec4p is recruited to accumulated exocytic vesicles upon overexpression of Sec15p (13, 21). This led us to speculate that Sec15 might be an effector for Rab11 in mammalian cells. To test this hypothesis, a quantitative yeast two-hybrid interaction assay was performed in which cells were cotransformed with a bait vector encoding Sec15 together with a prey vector encoding wild-type Rab4a, Rab6a, Rab7a, or Rab11a as indicated. Although Rab4, Rab6, and Rab7 localized to compartments in the perinuclear region that were clearly distinct from the GFP-Sec15-positive tubular/vesicular clusters, Xpress-Rab11 overlapped precisely with the exocyst subunit.

We used three independent biochemical approaches to investigate further the potential interaction between Sec15 and Rab11. First, an in vivo binding assay was performed in which COS-7 cells were cotransfected with expression vectors encoding Xpress-Sec15 and wild-type Rab11a or Rab4a fused to GST. Detergent extracts from the transfected cells were incubated in the presence of GTP and GDP, and the GST fusion proteins were affinity-purified on glutathione beads. The recovery of GST proteins and Xpress-Sec15 on the beads was assessed by immunoblotting (Fig. 10 B). When cells were cotransfected with Xpress-Sec15 and GST or GST-Rab4, the exocyst subunit was not detected in the bead eluates following incubation with GTP (Fig. 10 B, lanes 4 and 5). However, when the GST controls were substituted for GST-Rab11, a robust Xpress-Sec15 signal was detected in the bead eluate (Fig. 10 B, lane 7). In parallel incubations in which GTP was substituted for GDP, the recovery of Xpress-Sec15 was reduced significantly (Fig. 10 B, lane 8), consistent with a GTP-dependent interaction.

In addition, an in vitro binding assay was performed using purified histidine-tagged Rab proteins and GST-Sec15. In this experiment, GST-Sec15 was isolated from transfected COS-7 cells using glutathione beads. Bacterially expressed Hist-Rab11 or Hist-Rab4 was preincubated with GTPγS or GDPβS.
To confirm the interaction observed between the recombinant proteins, we investigated whether Rab11 associates with endogenous Sec15 (Fig. 10D). In this experiment, immobilized GST-Rab11 was preloaded with GDP\(\beta S\) or GTP\(\gamma S\) and incubated with a rat brain detergent extract. The beads were subsequently washed, and bound proteins were analyzed by immunoblotting using affinity-purified anti-Sec15 antibody. Sec15 was not detected on the beads when the GST-Rab11 fusion protein was substituted for control GST (Fig. 10D, lane 1). An ~3.5-fold increase in the level of endogenous Sec15 detected on the GST-Rab11 beads was observed when the GTPase was preloaded with GTP\(\gamma S\) compared with GDP\(\beta S\) (Fig. 10D, lanes 2 and 3). Thus, the GTP-dependent interaction between Rab11 and Sec15 was detected using multiple experimental approaches and is consistent with the colocalization data, indicating that the exocyst functions as a Rab11 effector complex in mammalian cells.

**DISCUSSION**

In this study, we have provided novel insights into the localization and function of Sec15. The association of overexpressed Sec15 with elements of the RE system is consistent with recent studies suggesting that in pancreatic acinar cells (27) and undifferentiated PC12 cells (28), exocyst subunits are associated with an uncharacterized perinuclear compartment that appears to be distinct from the Golgi apparatus. The observed localization of overexpressed Sec15 is also consistent with the demonstration that at least two distinct pathways from the TGN to the plasma membrane exist in yeast that are dependent on the exocyst subunit Sec6, one of which occurs via endosomes (29, 30).

Although the localization of Sec15 in mammalian cells is unclear, a previous study has shown that Sec6 colocalizes with tse045-VSVG in a subcompartment of the TGN in normal rat kidney cells (31). It is unclear therefore why Sec6 and Sec15 appear to localize to distinct intracellular compartments. One possibility we cannot exclude is that overexpressed Sec15 results in an abnormal phenotype that does not reflect the localization of the endogenous protein. However, the observations that Sec10 is recruited specifically to the Sec15-positive tubular-vesicular clusters and that recombinant Sec15 (like the endogenous protein) exhibits a GTP-dependent interaction with Rab11 suggest that the overexpressed Sec15 fusion proteins are functional. Furthermore, in *S. cerevisiae*, the specific effect of Sec15p overproduction (a block in secretion resulting from accumulation of exocytic vesicles) is consistent with the role of the endogenous protein in the secretory pathway based on genetic analysis (32).

There are a number of additional explanations for the finding that overexpressed Sec15 does not localize to the Golgi/TGN. First, Sec15 may function independently of other subunits of the exocyst. In this regard, it is noteworthy that, in yeast, Sec15p and Sec10p have been shown to exist in a separate subcomplex in addition to their presence in the exocyst (13). Furthermore, it has been shown previously that Sec10 fused to GFP does not colocalize with endogenous Sec6 or Sec8 and associates with an uncharacterized perinuclear compartment in polarized Madin-Darby canine kidney cells (19). Second, the role of exocyst subunits in plasma membrane-directed trafficking pathways may be cell type-specific. In support of this hypothesis, recent data have suggested that *Drosophila* Sec10 is not involved in polarized trafficking or exocytosis, but is essential for endocrine secretion (33). Third, Sec15 recruitment to Golgi/TGN compartments may involve a unique mechanism that does not lead to a detectable accumulation of TGN-derived transport intermediates in mammalian cells. Fourth, a family of Sec15-related proteins that function in distinct trafficking pathways may exist. Interestingly, a human protein...
directed trafficking pathways. The effector for all Rab proteins involved in plasma membrane-vacuolar transport (including Rab6a, or Rab7) is Rab4a, which regulates early endosomal trafficking (8, 9). We were unable to detect an interaction between Sec15 and Rab4a, or Rab7. Rab4a regulates early endosomal trafficking of proteins (pp75/Rip11, Rab11-FIP1, Rab11-FIP3/Eferin, Rab11-FIP4, and Rab-coupling protein (8, 9), that share a common 18–20-amino acid α-helical Rab11-binding domain. The primary sequence of Sec15 does not contain such a domain. There-fore, it is conceivable that Sec15 and Rab11-FIPs may bind simultaneously to Rab11, as recently demonstrated for myosin Vb and the Rab11-interacting protein Rab11-FIP2 (36). In con-trast to the strong interaction observed with Rab11, we were unable to detect an interaction between Sec15 and Rab4a, Rab6a, or Rab7. Rab4a regulates early endosomal trafficking and is involved in the degradative pathway from early to late endosomes as well as recycling from early endosomes to the plasma membrane (37, 38). The observation that Sec15 does not interact with Rab4a suggests that it does not function as an effector for all Rab proteins involved in plasma membrane-directed trafficking pathways.

Recent studies have indicated that the exocyst is an effector complex for at least six small GTPases in yeast and mammalian cells, including Sec4p, Rho1, Cdc42, Rho3, TC10, and Rap (39, 40). The observation that Sec15 is an effector for Rab11 raises the number of small GTPases that interact with the exocyst to seven. Rho1, Rho3, and Cdc42 are members of the Rho family of GTPases that regulate many cellular activities predominantly through their effects on the actin cytoskeleton. Thus, the exocyst is emerging as a central player in exocytic pathways that interact with multiple Ras-related small GTPases, including those that regulate vesicle trafficking (Rab proteins) as well as the actin cytoskeleton (Rho family). Under-