TITLE: Sec15 is an Effector for the Rab11 GTPase in Mammalian Cells

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SUMMARY

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 which 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 are 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 Rabs4, 6 or 7. These findings provide the first evidence that the exocyst functions as a Rab effector complex in mammalian cells.
INTRODUCTION

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 Rabs 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 (GEFs) and GTPase activating proteins (GAPs), respectively. In the GTP-bound state, Rabs 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 Rabs regulate a diverse array of effector molecules, including factors that promote vesicle formation, motility, docking and fusion (3). GAP-catalyzed GTP hydrolysis results in the dissociation of effector complexes and extraction of the 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 MDCK cells (6), and trans Golgi network (TGN) to plasma membrane trafficking via REs in BHK cells (7). Several Rab11 effectors have been described that are involved in recycling, including Rab11BP/Rabphilin-11, pp75/Rip11, myosin Vb, Rab11-FIP1, Rab11-FIP3/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 *S. 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**

A polyclonal anti-Sec15 antibody was prepared by immunization of rabbits with a C-terminal peptide (KDTSKKNNIFAQFRKNDRDQRKC) conjugated to diptheria toxin. The antibody was affinity-purified from the antiserum using the same peptide immobilized on Amino Link Plus Coupling Gel (Pierce). Anti-Xpress mouse monoclonal antibody and Lipofectamine transfection reagent were purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-glutathione S-transferase (GST) and anti-Rab11 antibodies were from Zymed Laboratories (South San Francisco, CA). Anti-green fluorescent protein (GFP) monoclonal antibody, Alexa Fluor-conjugated secondary antibodies and Texas Red Tfhn were purchased from Molecular Probes (Eugene, OR). Monoclonal anti-Sec6 (clone 9H5) was from Stressgen (Victoria, Canada). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

**Construction of Expression Plasmids**
The clones encoding rSec10 and rSec15 were obtained from Dr. R. Scheller (Stanford University, Stanford, CA), and human TfnR from Drs. M. Silverman and G. Banker (Oregon Health Sciences University, Portland, OR). Complementary DNAs encoding wild type Sec15, Sec10 (amino acids 2-822 for Sec15, and 2-707 for Sec10) and TfnR were amplified by PCR, and subcloned into the pCR-Blunt TOPO shuttle vector (Invitrogen, Carlsbad, CA). The cDNA clones encoding wild type Rab4a, Rab6a, Rab7 and Rab11a 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, Rab11(S25N) and Rab11(Q70L) were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. For construction of expression vectors the cDNAs were inserted in-frame into the following plasmids: pcDNA4 HisMax C (Invitrogen, Carlsbad, CA) for Xpress-tagged proteins; pEGFP-C (Clontech Laboratories, Palo Alto, CA) for GFP-fusion proteins; pEBG (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

COS-7, CHO and 293 cells were cultured at 37°C in DMEM supplemented with 10% FBS (CSL Biosciences, Parkville, VIC, Australia) and 100 units/ml of penicillin and streptomycin. Transfection of CHO cells was performed using the Lipofectamine reagent (Invitrogen, Carlsbad, CA) 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 µl mixture containing plasmid DNA (15 µg each plasmid) in 0.15 M NaCl, and electroporated at 200 V, 975 µF. For microscopy experiments, the cells were plated onto glass coverslips and allowed to recover for 16-20 hrs at 37°C. The transfected cells were washed three times with PBS, fixed with 3% paraformaldehyde for 20 min at room temperature and permeabilized in 100% methanol at -20°C for 2 min. Following washing with PBS, the cells were incubated with blocking buffer (5% goat serum, 0.01% Triton X-100 in PBS) for 30 min. Binding of primary antibodies was performed for 1 hr at room temperature in blocking buffer at the following dilutions: anti-Xpress (1:500), anti-Rab11 (1:20). The cells were washed three times with PBS, and incubated with Alexa-conjugated anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) at a dilution of 1:300 in blocking buffer for 1 hr at room temperature. The coverslips were washed three times with PBS and mounted onto glass slides. Single plane images were obtained using a Leica TCS-NT confocal microscope (100 x objective).

**Transferrin Uptake and Recycling Assays**

COS-7 cells were cotransfected with expression vectors encoding GFP-Sec15 and Xpress-TfnR and plated onto coverslips. Twenty hours post-transfection, the cells were washed once with DMEM, and serum-starved for 2 hr by incubation at 37°C in DMEM supplemented with 0.1% BSA. The cells were pulsed with Texas Red-labelled Tfn (20 µg/ml) for 5 min at 37°C, washed twice with PBS on ice, and chased with holo-Tfn (200 µg/ml) for increasing time points. The cells were placed on ice to terminate trafficking, fixed and permeabilized as described above, and stained using Extravidin-TRITC (1:100) to detect internalized biotin-Tfn.
Binding Assays

For in vivo binding assays, COS-7 cells were transfected with expression vectors as indicated. Twenty hours after transfection, confluent 10 cm dishes of transfected cells were lysed in 1 ml of lysis buffer (1 % Triton X-100, 0.15 M NaCl, 5 mM EDTA, 1mM dithiothreitol, 25 mM Tris-HCl, pH 7.4, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml AEBSF, 10 µg/ml aprotinin). Lysates (300 µl) were incubated with 1 mM GTPγS or GDPβS for 20 min at room temperature, and supplemented with 10 mM Mg(Cl)₂. GST fusion proteins were recovered by incubation with 15 µl of glutathione beads for 2 hr at 4°C. For in vitro binding assays purified His₆-Rabs were pre-loaded with 1 mM GTPγS or GDPβS in binding buffer (0.15 M NaCl, 5 mM EDTA, 25 mM Tris-HCl, pH 7.4) for 20 min at 37°C. Detergent lysates from cells expressing GST-Sec15 were incubated with glutathione beads for 4 hrs at 4°C to immobilize the GST fusion protein. The GST-Sec15 beads were washed three times with binding buffer, and incubated for a further 2 hrs in the same buffer supplemented with 10 mM Mg(Cl)₂ and 0.8 µM His₆-Rab11 with bound GTPγS or GDPβS. To investigate binding between endogenous Sec15 and GST-Rab11, whole rat brain tissue was washed twice in ice-cold PBS, and homogenized in lysis buffer. The detergent extract was centrifuged at 14,000g for 20 min, and the supernatant pre-cleared by incubation with glutathione beads for 30 min. Immobilized GST-Rab11 or GST control were pre-incubated with 1 mM GTPγS or GDPβS in binding buffer for 20 min at 37°C, and washed in the same buffer. The pre-cleared lysates were supplemented with 10 mM Mg(Cl)₂, and incubated with immobilized GST proteins (0.2 µM) for 2 hrs at 4°C. The beads were washed three times with 0.15 M NaCl, 2.5 mM Mg(Cl)₂, 25 mM
Tris-HCl, pH 7.4, and bound proteins eluted by boiling in SDS gel loading buffer. Samples were subjected to SDS-PAGE, and immunoblots probed using anti-His (1:1,000), anti-Xpress (1:5,000), anti-GST (1:1000), or affinity-purified anti-Sec15 (1:500) primary antibodies followed by HRP-conjugated secondary antibodies. The blots were developed using ECL, and quantitated by gel densitometry.

**Yeast Two-Hybrid Interaction Assays**

The two-hybrid assay was performed as described previously (15). The cDNAs encoding Sec15 and the Rabs were ligated into the vectors pEG202 (bait) and pJG4-5 (prey), respectively. Yeast strain EGY48r cells were cotransformed with the plasmids, and plated onto synthetic media lacking uracil, tryptophan and histidine. The transformed cells were subsequently plated onto media containing X-Gal as substrate and galactose to induce expression of the prey fusion protein. The binding affinities were quantitated in triplicate using a beta-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 hrs at 37°C. Subsequently, the medium was supplemented with 25 µg/ml human Tfn conjugated to peroxidase (Tfn-HRP; Rockland Inc., Gilbertsville, PA) for 30 min at 37°C. The DAB reaction was performed as described previously (16). Following the DAB reaction, the cells were placed on ice, washed 3 times with PBS, and fixed using 2 % paraformaldehyde, 0.05 % glutaraldehyde in 0.08 M Sörensen’s buffer for 1 hr at
0°C. The fixed cells were washed twice with 0.08 M Sörensen’s buffer supplemented with 5 % (w/v) sucrose, scraped from the dishes and pelleted by centrifugation. Ultrathin cryosections were prepared using a modified Tokyasu technique (17). Immunolabeling of the cryosections was performed using 4 ug/ml of a mouse monoclonal anti-GFP antibody (Molecular Probes), or an equivalent concentration of a mouse monoclonal anti-\textit{A. niger} IgG1 isotype control (DAKO), 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 (GFP-Sec15) in transfected cells (Fig. 1). Initial immunoblotting experiments confirmed that the expression vectors directed the synthesis of Sec15 polypeptides of the appropriate molecular weight (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. 1A 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 from 4 hrs up to at least 48 hrs. 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. 1C 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).

Initially, we examined the effect of the microtubule (MT)-depolymerizing agent nocodazole on the localization of GFP-Sec15 in transiently transfected COS-7 cells (Fig. 2). The perinuclear localization of Golgi cisternae, endosomal compartments and lysosomes is maintained by tethering to MTs. Following nocodazole treatment (100 µM for 30 min) the dots of fluorescence became dispersed throughout the cytoplasm, whereas in control cells treated with vehicle (0.3% DMSO) their perinuclear localization was maintained (Fig. 2). These findings suggested that the observed localization of Sec15 resulted from its association with a MT-tethered compartment(s) or vesicle cluster, and not from non-specific 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 MTs.

Recent studies have revealed that Sec10 associates directly with Sec15 in yeast and mammalian cells (13,19). Therefore, we examined whether Sec10 is 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-tagged Sec10 (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 are implicated in the trans Golgi network (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 (ER) at the restrictive temperature (39.5°C), and is able to exit the ER and traffic to the plasma membrane following incubation at the permissive
However, if the cells are shifted directly from the restrictive temperature to 19°C, ts045-VSVG is transported from the ER and subsequently arrested in Golgi/TGN compartments. Therefore, cells expressing ts045-VSVG-GFP and Xpress-Sec15 were incubated at 39.5°C for 20 hrs followed by 19°C for 2 hrs to trap 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 untagged VSVG in COS cells (20).

Following temperature shift from 39.5°C to 19°C for 2 hrs, a patch of ts045-VSVG-GFP fluorescence was detected in COS-7 cells, characteristic of the Golgi (Fig. 4A). As expected, Xpress-Sec15 labelled punctate structures that were concentrated in the perinuclear region in cotransfected cells (Fig. 4B). Merged images (Fig. 4C 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 the Sec15 and ts045-VSVG in the perinuclear region rather than co-association on Golgi/TGN membranes since the structures labelled 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 hrs 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. 4E-H). At late time points a rim of 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 labelled by the exocyst subunit are accessible to endocytic cargo. The transferrin (Tfn) recycling pathway has been studied extensively in mammalian cells. Following its binding to the Tfn receptor (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 recycling endosome (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 the human TfnR (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 where the cotransfected cells were incubated in the presence of biotinylated Tfn for 5 min at 37°C (pulse), then chased with unlabelled 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 labelled dispersed punctate structures that were characteristic of early endosomes, and there was little colocalization with GFP-Sec15. The rapid internalization of Tfn suggested 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 (20 and 30 min) time points substantial overlap was observed. Indeed,
after a chase time of 30 min the punctate perinuclear structures that were labelled 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 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 recycling endosome (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) or LAMP2 (late endosomes/lysosomes). We found that while the compartments labelled 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 immunoelectron 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 labeled with electron dense peroxidase-DAB 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 endosome 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 where COS-7 cells expressing GFP-Sec15 (or GFP control) and hTfnR were incubated with Texas Red-labelled 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.
the 40 min chase, the intracellular Tfν 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 Tfν 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 Tfν 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. While some overlap between Rab4a, Rab6a or Rab7 and Sec15 was apparent, it was clear that their localizations 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 where 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 where cells were cotransformed with a bait vector encoding Sec15 together with prey vectors
encoding wild type Rab4a, Rab6a, Rab7, Rab11a, the GDP-restricted mutant, Rab11(S20N), or the GTP-restricted mutant, Rab11(Q70L). Immunoblotting analysis of lysed cells revealed that all Rabs tested were expressed at roughly equal levels (data not shown). The strength of the interaction was determined using a β-galactosidase reporter assay (Fig. 10A). Yeast cells coexpressing Sec15 and wild type Rab11 exhibited a more than seven-fold higher β-galactosidase activity than those coexpressing Sec15 and the other Rabs. Furthermore, the strength of the interaction was reduced significantly with the Rab11(S25N) mutant, and increased more than five-fold with Rab11(Q70L) relative to the wild type protein, consistent with the hypothesis that Sec15 functions as an effector for Rab11.

We used three independent biochemical approaches to investigate further the potential interaction between Sec15 and Rab11. First, an in vivo binding assay was performed where 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 GTPγS or GDPβS, and the GST fusion proteins affinity-purified on glutathione beads. The recovery of GST proteins and Xpress-Sec15 on the beads was assessed by immunoblotting (Fig. 10B). 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 GTPγS (Fig. 10B; 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. 10B; lane 7). In parallel incubations where GTPγS was substituted for GDPβS the recovery of Xpress-Sec15 was reduced significantly (Fig. 10B; lane 8), consistent with a GTP-dependent interaction.
In addition, an in vitro binding assay was performed using purified histidine-tagged Rabs and GST-Sec15 fusion protein (GST-Sec15). In this experiment, GST-Sec15 was isolated from transfected COS-7 cells using glutathione beads. Bacterially expressed His\textsubscript{6}-Rab11 or His\textsubscript{6}-Rab4 were preincubated with GTP\textsubscript{γS} or GDP\textsubscript{βS}, incubated with immobilized GST-Sec15, and the beads washed extensively. His\textsubscript{6}-Rab11 was recovered on the beads when incubation was performed with GTP\textsubscript{γS}, but not GDP\textsubscript{βS} (Fig. 10C, lanes 2 and 3). In contrast, the control His\textsubscript{6}-Rab4 protein was not detected in the bead eluates following incubation with GDP\textsubscript{βS} or GTP\textsubscript{γS} (Fig. 10B, lanes 6 and 7).

In order 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\textsubscript{βS} or GTP\textsubscript{γS} and incubated with a rat brain detergent extract. The beads were subsequently washed and bound proteins analyzed by immunoblotting using affinity-purified Sec15 antibody. Sec15 was not detected on the beads when the GST-Rab11 fusion protein was substituted for GST control (Fig. 10D, lane 1). A roughly 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\textsubscript{γS} compared to GDP\textsubscript{βS} (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 provide 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 plasma membrane exist in yeast that are dependent on the exocyst subunit Sec6, one of which occurs via endosomes (29,30).

While the localization of Sec15 in mammalian cells is unclear, a previous study has shown that Sec6 colocalizes with ts045-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 observation 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 suggests 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 MDCK 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 may exist that function in distinct trafficking pathways. Interestingly, a human protein KIAA0919 has recently been described that shares 66% identity with the human orthologue of Sec15, and is a component of a variant of the exocyst complex that interacts with the small GTPase RalA (34).

What are the punctate Sec15-positive structures detected by fluorescence microscopy? Analysis at the ultrastructural level revealed that the overexpressed protein associates with clusters of 50-75 nm diameter tubular/vesicular profiles that contain internalized Tfn. This is similar to the previously reported tubular/vesicular morphology of the RE compartment in CHO cells (35). However, the punctate localization of overexpressed Sec15 appeared distinct from the morphology of the pericentriolar RE compartment, which is typically detected as a single concentrated region of fluorescence in mammalian cells (5). This may result from an effect of Sec15 overexpression on the
morphology of the RE compartment. Alternatively, the tubular-vesicular clusters may represent RE-derived intermediates in the endocytic recycling pathway that accumulate upon Sec15 overexpression. Interestingly, the Tfn-positive tubular-vesicular clusters are highly reminiscent of the patches of accumulated secretory vesicles observed upon overproduction of Sec15p in *S. cerevisiae* (13,21). The formation of the secretory vesicle clusters is believed to reflect a normal association between Sec15p and the vesicles since it is dependent on the function of the Rab GTPase Sec4p, and its nucleotide exchange factor, Sec2p.

We have provided the first demonstration that Sec15 functions as an effector molecule for a Rab GTPase in mammalian cells. Recent studies have identified a group of five Rab11 effectors, 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 (Rab11BD/RBD11). The primary sequence of Sec15 does not contain such a domain. Therefore, 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 contrast to the strong interaction observed with Rab11, we were unable to detect an interaction between Sec15 and Rabs 4a, 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 Rabs 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 Ral (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 interacts with multiple ras-related small GTPases, including those that regulate vesicle trafficking (Rabs) as well as the actin cytoskeleton (Rho family). Understanding how these interactions are coordinated represents a key challenge for future research.

REFERENCES

FOOTNOTES

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Abbreviations: GFP, green fluorescent protein; GST, Glutathione S-transferase; MT, microtubule; RE, recycling endosome; TfnR, transferrin receptor.

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FIGURE LEGENDS

Fig. 1. 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 hrs after transfection, and examined by confocal fluorescence microscopy. Single optical sections of transfected cells are shown.

Fig. 2. The Sec15-positive punctate structures are dispersed upon treatment with the MT-depolymerizing agent nocodazole. COS-7 cells expressing GFP-Sec15 were treated in the absence (-noc) or presence (+noc) of 100 µM nocodazole for 30 min at 37°C. The control
cells were incubated with an equivalent concentration of the solvent (0.3% DMSO) prior to examination by confocal microscopy.

**Fig. 3.** Sec10 is recruited to the Sec15-positive structures. COS-7 cells were transfected with expression vectors encoding Xpress-tagged Sec10 alone (A), GFP-Sec15 and Xpress-Sec10 (B), or GFP-Sec15 alone (C). For visualization of Sec10 and Sec6, the fixed cells were incubated with mouse anti-Xpress and anti-Sec6 (clone 9H5) monoclonal antibodies, respectively, followed by Alexa Fluor 568 conjugated anti-mouse secondary antibody.

**Fig. 4.** Sec15 does not colocalize with the exocytic cargo molecule VSVG. Transiently transfected COS-7 cells expressing Xpress-Sec15 and ts045-VSVG-GFP were incubated at 39.5°C for 20 hrs followed by 19°C for 2 hrs to trap VSVG in the Golgi/TGN. The cells were subsequently transferred to ice (A-D), or shifted to 32°C for 30 min (E-H) prior to fixation and processing for microscopy. The localizations of ts045-VSVG-GFP (A and E) and Xpress-Sec15 (B and F) together with merged images (C and G) in cotransfected cells are shown. (D) and (H) are higher magnification views of the Golgi regions shown in (C) and (G), respectively.

**Fig. 5.** Sec15 colocalizes with internalized Tfn. COS-7 cells were cotransfected with expression vectors encoding GFP-Sec15 and the hTfnR. Subsequently, a pulse-chase experiment was performed where the cells were incubated in the presence of biotinylated Tfn for 5 min at 37°C (pulse), washed and chased with unlabelled Tfn for the indicated
time points. The cells were fixed and permeabilized, and the localization of GFP-Sec15 and endocytosed biotinylated Tfn examined by confocal fluorescence microscopy.

**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 DAB 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. Size bar = 200 nm.

**Fig. 8.** Intracellular accumulation of internalized Tfn in cells overexpressing Sec15. A pulse-chase experiment was performed where COS-7 cells expressing the hTfnR as well as GFP-Sec15 or GFP control were pulsed with Texas Red-Tfn for 5 min at 37°C. The cells were subsequently placed on ice (0 min) or chased with excess holo-Tfn for 40 min.
as indicated. The transfected cells were fixed and permeabilized, and analysed for retention of internalized Tfn by confocal microscopy.

**Fig. 9.** Sec15 colocalizes with the RE marker Rab11. COS-7 cells were cotransfected with expression vectors encoding GFP-Sec15 and Xpress-tagged Rab4, Rab6, Rab7 or Rab11 as indicated. While Rabs 4, 6 and 7 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.

**Fig. 10.** Sec15 is a Rab11 effector molecule. (A) Interaction between Sec15 and Rab11 assessed using the yeast two-hybrid system. Yeast cells were cotransformed with a bait vector encoding Sec15 and prey plasmids encoding wild type (WT) Rabs 4, 6, 7, 11, the Rab11 GDP-restricted mutant, Rab11(S25N), or the Rab11 GTP-restricted mutant, Rab11(Q70L). The strength of the interaction was determined in triplicate using a β-galactosidase reporter assay. The mean values (indicated above each bar) and standard deviation are shown for each sample. (B) In vivo binding assay where detergent lysates were prepared from COS-7 cells expressing Xpress-Sec15, and GST control, GST-Rab4a or GST-Rab11a. Following incubation with GTPγS or GDPβS, the GST proteins were recovered from the lysates on glutathione beads and associated Xpress-Sec15 analyzed by immunoblotting. Lanes 1-3 are the lysates from cells transfected with GST control (lane 1), GST-Rab4a (lane 2), or GST-Rab11a (lane 3). Lanes 4-8 are the complexes recovered on the beads following incubation of GST control lysate with GTPγS (lane 4); GST-Rab4 lysates with GTPγS (lane 5) or GDPβS (lane 6), and GST-Rab11 lysates with
GTPγS (lane 7) or GDPβS (lane 8). (C) In vitro binding assay where immobilized GST-Sec15 was incubated with purified His₆-Rab11a (lanes 2 and 3) or His₆-Rab4 (lanes 6 and 7) in the presence of GDPβS (lanes 2 and 6) or GTPγS (lanes 3 and 7) prior to immunoblotting analysis. Lanes 1 and 5 are GST controls, while lanes 4 and 8 are His₆-Rab11a and His₆-Rab4 controls, respectively. (D) GTP-dependent interaction of endogenous Sec15 with Rab11. Immobilized GST-Rab11 (lanes 2 and 3) or GST control (lane 1) were pre-incubated with GDPβS or GTPγS followed by a rat brain detergent extract as a source of endogenous Sec15. After the binding step, the beads were washed and bound proteins analyzed by immunoblotting using the affinity-purified Sec15 antibody. GST fusion proteins were detected by Ponceau S staining of the PVDF membrane (lower panel). All blots were developed using ECL. These experiments were repeated a minimum of three times and representative data are shown.
Fig. 2
Fig. 3
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 9
Fig. 10

A

\[ \text{β-Galactosidase activity (arbitrary units)} \]

B

1 2 3 4 5 6 7 8

Xpress
Sec15

GST-Rab

C

1 2 3 4 5 6 7 8

His6-Rab

GST-Sec15

D

GβS GγS GβS

Sec15

GST-Rab11
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