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## Ral GTPases Regulate Exocyst Assembly through Dual Subunit Interactions\*

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Ral GTPases have been implicated in the regulation of a variety of dynamic cellular processes including proliferation, oncogenic transformation, actin-cytoskeletal dynamics, endocytosis, and exocytosis. Recently the Sec6/8 complex, or exocyst, a multisubunit complex facilitating post-Golgi targeting of distinct subclasses of secretory vesicles, has been identified as a *bona fide* Ral effector complex. Ral GTPases regulate exocyst-dependent vesicle trafficking and are required for exocyst complex assembly. Sec5, a membrane-associated exocyst subunit, has been identified as a direct target of activated Ral; however, the mechanism by which Ral can modulate exocyst assembly is unknown. Here we report that an additional component of the exocyst, Exo84, is a direct target of activated Ral. We provide evidence that mammalian exocyst components are present as distinct subcomplexes on vesicles and the plasma membrane and that Ral GTPases regulate the assembly interface of a full octameric exocyst complex through interaction with Sec5 and Exo84.

High fidelity targeting of secretory vesicles to discrete plasma membrane domains is critically important for a diverse array of cellular activities. These include establishment and maintenance of cell polarity, synapse formation and synaptic plasticity, controlled release of neurotransmitters, enzymes, hormones, and cytokines, and regulation of levels of plasma membrane receptors, transporters, and lipids. Although much is known about the cellular machinery responsible for docking and fusion of secretory vesicles once at the plasma membrane, far less is understood about mechanisms responsible for targeting secretory vesicles to specific plasma membrane domains (6–9). Multiple cellular machines are likely required for sorting, targeting, and tethering of discrete classes of secretory vesicles. One these, the Sec6/8 complex or exocyst, has emerged as a core facilitator of vesicle delivery to sites of rapid membrane expansion (10), of membrane remodeling (11), and to subsets of specialized membrane domains (12).

The exocyst is a multi-protein complex conserved from yeast to humans that includes Sec3, Sec5, Sec6, Sec8, Sec10, Sec15,

Exo70, and Exo84 (10, 11). In budding yeast, this complex is essential for exocytosis and has been shown to direct vectorial targeting of Golgi-derived secretory vesicles to the growing bud-tip or to the developing mother/bud septum (10). In mammalian cells, the Sec6 and Sec8 components of the exocyst have been localized to lateral plasma membranes near the tight junctions of polarized epithelial cells (12), in sites of developing synapses of neuronal cells, and in growth cones of differentiating PC12 cells (11). Like yeast, the mammalian exocyst has been directly implicated in post-Golgi targeting of secretory vesicles to discrete membrane sites (11). For example, blocking exocyst complex formation by delivery of neutralizing antibodies to permeabilized epithelial cells resulted in mis-sorting of basolateral membrane proteins to the apical surface without affecting the appropriate spatial delivery of apical membrane proteins (12).

The mechanism by which the exocyst promotes vesicle targeting has not been resolved. Work in yeast suggests that the exocyst is required for vesicle tethering before assembly of exocytic SNARE (soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors) complexes and membrane fusion. A subset of exocyst subunits likely forms a targeting patch on the plasma membrane, whereas a sub-complex acts to bridge vesicles with the targeting patch (10, 13). The targeting and assembly of these complexes is dynamically regulated by Sec4 and Rho1 GTPases in yeast through discrete subunit interactions (10, 13, 14). Ral (Ras like) GTPases have recently been defined as critical components of the cellular machinery regulating post-Golgi targeting of distinct subsets of secretory vesicles in mammalian cells through direct interaction with the exocyst (1). Activated Ral proteins bind the Sec5 subunit of the exocyst, and appropriate regulation of this interaction is apparently required for exocyst function (1, 15–17). A mechanistic understanding of the contribution of Ral GTPases to exocyst function remains to be developed, but clues come from observations that Ral proteins are located on plasma membrane domains and secretory vesicles, and Ral expression is required for appropriate assembly of the full octameric exocyst complex (1).

Here we show that Ral GTPases regulate exocyst assembly through direct interactions with two distinct subunits of the exocyst complex, Sec5 and Exo84. Like Sec5, the interaction of Ral with Exo84 is direct and selective for the GTP-bound “activated” conformation of the GTPase. Remarkably, the Ral binding domain of Exo84 completely overlaps with a pleckstrin homology (PH)<sup>1</sup> domain, and RalGTP competes with phosphatidylinositol 3,4,5-trisphosphate for interaction with Exo84.

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<sup>1</sup> The abbreviations used are: PH, pleckstrin homology; GTPγS, guanosine 5′-3-O-(thio)triphosphate; siRNA, small interfering RNA; PI3,4,5P3, phosphatidylinositol (PI) 3,4,5-trisphosphate; GST, glutathione S-transferase.

Cell fractionation data and analysis of exocyst complex assembly suggests that, as in yeast, a significant fraction of Sec5 and Exo84 is present in distinct subcomplexes. We propose that Ral GTPases function at the interface between these subcomplexes through dual effector interactions to regulate assembly of the full exocyst complex during a vesicle-targeting event.

#### MATERIALS AND METHODS

**Plasmids**—pGAD352X-Exo84 and pGAD352X-Exo84-(122–333) encode full-length and the Ral binding domain of human Exo84 as SpeI/XhoI fragments in pGAD352X. pGEX4T-2-Exo84-(122–333) was derived from the pGAD insert as a blunt SpeI/XhoI fragment in the SmaI/XhoI sites of pGEX4T-2 vector. pGEX4T-3-Exo84 contains the full Exo84 coding sequence as an EcoRI/NotI fragment. PCAN-MycExo84 contains the full Exo84 coding sequence as a blunt SpeI/NotI fragment in the EcoRV/NotI sites of pCan-Myc. The remaining plasmids used in this study are as described previously (1).

**Antibodies and Other Materials**—Exo84 and Sec5 rabbit polyclonal antibodies were raised against peptide antigens (BioCarta). The antibodies were validated using whole cell lysates expressing Myc-tagged Exo84 or hemagglutinin-tagged Sec5 and by using lysates from cells treated with siRNA targeting Exo84 or Sec5. Commercial mouse monoclonal anti-synaptophysin (clone SVP-38) (Sigma S5768) and mouse monoclonal anti-Na,K-ATPase  $\alpha$ -1 (Upstate Biotechnology 05-369) were used. The remaining antibodies are as described previously (1).

**Phosphatidylinositol (PI) 3,4,5-trisphosphate, dipalmitoyl (PI3,4,5P3), phosphatidylinositol 4,5-bisphosphate, dipalmitoyl, phosphatidylinositol 3,4-bisphosphate, dipalmitoyl, and phosphatidylinositol 3-phosphate, dipalmitoyl** were obtained from Matreya, Inc. **L- $\alpha$ -Phosphatidylinositol 4-phosphate (brain), L- $\alpha$ -phosphatidylinositol 4,5-bisphosphate (brain), L- $\alpha$ -phosphatidylinositol (liver), L- $\alpha$ -phosphatidylcholine (egg or brain), and L- $\alpha$ -phosphatidylserine (brain)** were obtained from Avanti Polar Lipids Inc. **L-3-Phosphatidyl[N-methyl- $^3$ H]choline, 12-dipalmitoyl** (specific activity, 112 mCi/mg) was from Amersham Biosciences. Nycodenz and sucrose were obtained from Sigma. siRNAs targeting Exo84 were synthesized from 5'-GGUGCCACUUUACUCUAUAdTdT-3' and 5'-UUAAGAG-UAAAGUGGCCACcTdT-3'.

**Cell Culture and Transfection**—293T cells were maintained in sodium pyruvate-free Dulbecco's modified Eagle's medium cell culture medium (Invitrogen) with 10% fetal bovine serum (Invitrogen). Transfection of 293T cells was performed using calcium phosphate precipitation as previously described (1). PC12 cells were maintained in RPMI1640 medium with 10% horse serum (heat-inactivated) and 5% fetal bovine serum on collagen-coated dishes. Cells were transfected using Pfx-1 according to the manufacturer's instructions (Invitrogen). HeLa cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. siRNAs were transfected with Oligofectamine (Invitrogen) according to the manufacturer's optimized protocol.

**Yeast Two-hybrid Analysis**—Two-hybrid screens and pairwise mating assays were performed using standard procedures (2).

**Protein Complex Analysis**—*In vitro* binding assays and coimmunoprecipitation assays were performed as previously described (1).

**Preparation of  $^3$ H-Labeled Liposomes**—Phospholipids (total of 1.75 mg) dissolved in chloroform were mixed in a glass tube and dried under a nitrogen stream. Liposomes were made from 1.25 mg of phosphatidylcholine (Avanti Polar Lipids) mixed with 0.5 mg of phosphatidylserine, PI, L- $\alpha$ -phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate (Avanti Polar Lipids), phosphatidylinositol 3,4,5-trisphosphate, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3-phosphate (Matreya). Liposomes also contained 20  $\mu$ Ci of L-3-phosphatidyl[N-methyl- $^3$ H]choline, 12-dipalmitoyl (Amersham Biosciences, specific activity, 112 mCi/mg) as a tracer. Dried phospholipids were dissolved in 10 ml of hydration buffer (50 mM HEPES, pH 7.1, 100 mM NaCl). After 40 min of incubation in hydration buffer lipids were re-suspended by vigorous shaking for 1 min. The resulting suspensions were sonicated for 30 s (VibraCell, Sonics and Materials) at 70% energy output and briefly centrifuged (700  $\times g$  for 5 min) to remove aggregates. Liposome suspensions were stored on ice and used within 2 weeks.

**Phospholipid Binding Assays**—Liposome precipitation assays were performed essentially described by Davletov and Sudhof (3). 400 pmol of GST-Exo84-(122–333) immobilized on glutathione-agarose beads were prewashed and re-suspended in 0.2 ml of hydration buffer containing  $^3$ H-labeled liposomes (17.5  $\mu$ g of lipids;  $\sim$ 120,000 cpm). The mixture was incubated at room temperature with vigorous shaking for 21 min. The beads were washed 3 times with hydration buffer and incubated

with 10% SDS for 5 min. Liposome binding was then quantified by liquid scintillation counting. To assess the consequences of Ral on the PH/phosphoinositide interactions, recombinant His<sub>6</sub>-Ral was eluted from nickel-agarose beads and loaded with GDP or GTP $\gamma$ S. *In vitro* binding assays using 400 pmol of GST-Exo84-(122–333) and 2400 pmol of Ral were performed as described (1). Phospholipid binding measurements were performed as described above. To assess the association of Exo84 with liposomes by density gradient fractionation, a modification of the procedure described by Matsuoka *et al.* (4) was used. Lipids were hydrated with 65% Nycodenz buffer (20 mM HEPES, pH 7.1, 50 mM potassium acetate, 10% glycerol, 65% Nycodenz (Sigma)). Liposomes, prepared as described above, were made with a ratio of 90:30:10 phosphatidylcholine:phosphatidylserine:phosphoinositide (PI, phosphatidylinositol 4,5-bisphosphate, or phosphatidylinositol 3,4,5-trisphosphate). 1  $\mu$ g of recombinant GST-Exo84-(122–333) was eluted from Sepharose beads and mixed with 30  $\mu$ l of liposomes, giving a final Nycodenz concentration of 50%. Binding reactions were incubated 15 min on ice and then overlaid with 61  $\mu$ l of 45% Nycodenz buffer, 77  $\mu$ l of 40% Nycodenz buffer, and 14  $\mu$ l of 0% Nycodenz buffer. The resulting step gradient was centrifuged in a Beckman MLA-130 rotor for 3 h at 70,000 rpm. 30- $\mu$ l fractions were collected from the top of the tube and analyzed by Western blotting.

**Immunofluorescence**—PC12 cells were plated on poly-L-lysine (Sigma; P-1399)-coated coverslips and transfected as described previously (1). At 24 h post-transfection cells were incubated in reduced serum medium (0.5% horse serum) for 24 h. At 48 h post-transfection, cells were washed in low potassium saline (145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5.6 mM glucose, 15 mM HEPES, pH 7.4). Cells were then incubated in low potassium saline or high potassium saline (95 mM NaCl, 56 mM KCl, 2.2 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5.6 mM glucose, 15 mM HEPES, pH 7.4) at 37 °C followed by fixation with 3.7% paraformaldehyde in phosphate-buffered saline. Fixed cells were permeabilized with 0.25% Triton X-100 and 1% bovine serum in phosphate-buffered saline. For the detection of Exo84 Ral binding domain, cells were incubated with anti-Myc 9E10 antibodies (1:75 dilution) followed by a 1:750 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (The Jackson Laboratory, Bar Harbor, ME). Fluorescence images were acquired on a Leica inverted laser-scanning confocal microscope equipped with a multiband confocal imaging spectrophotometer.

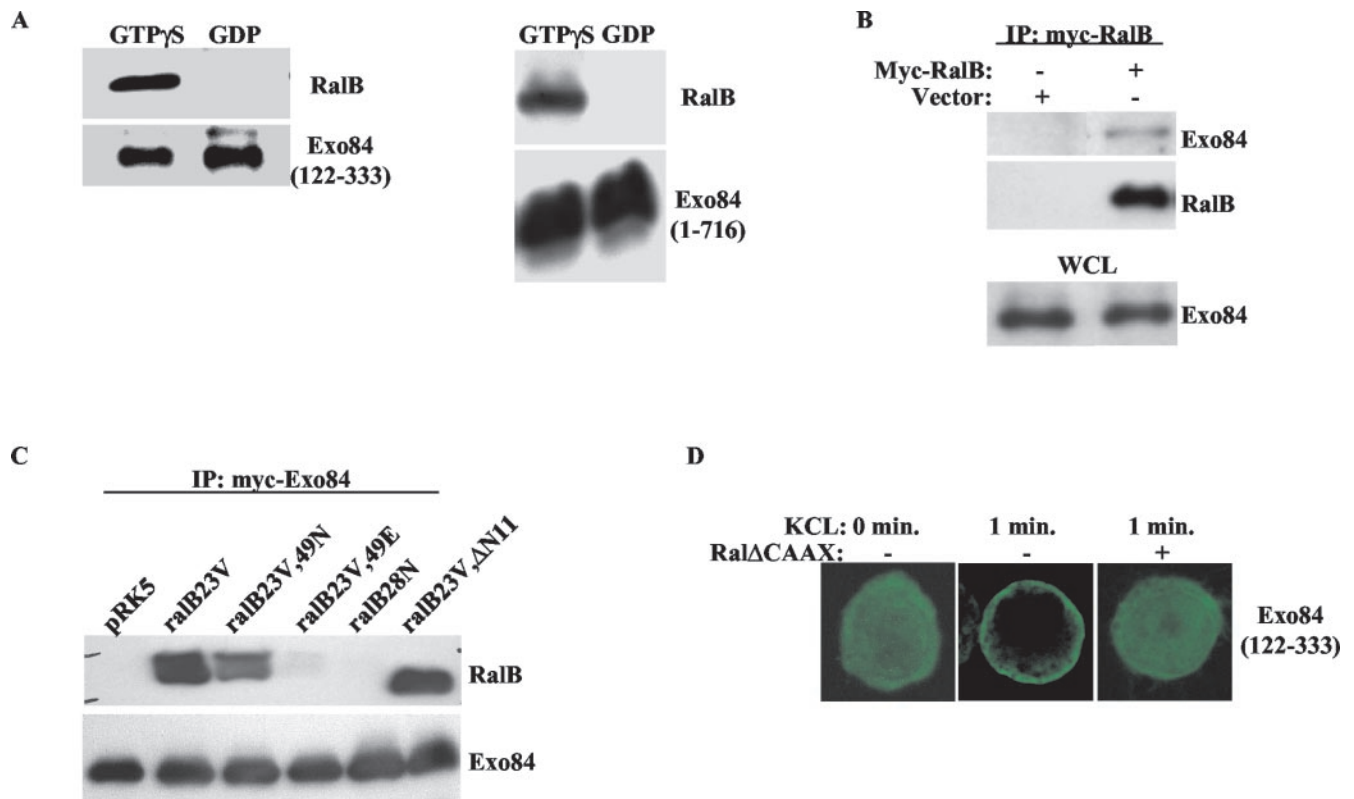
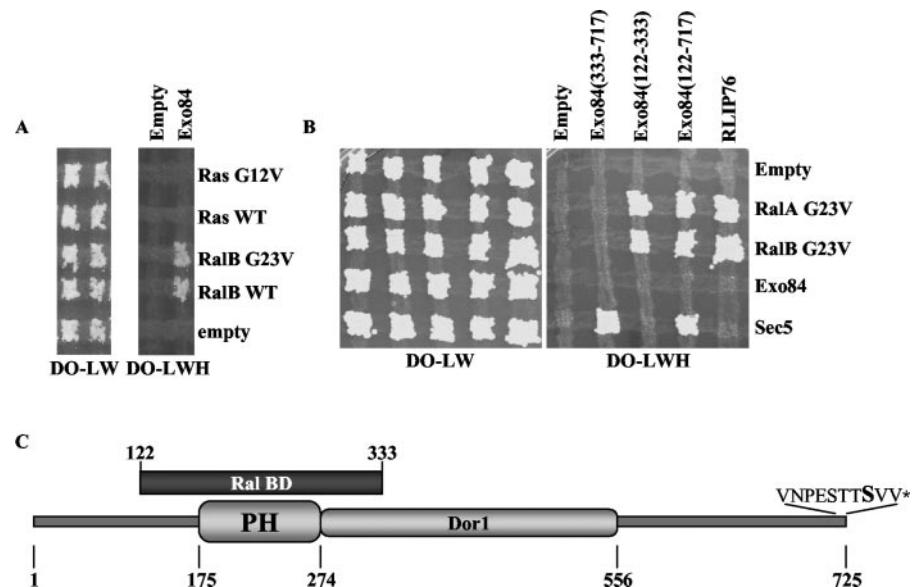
**Density Gradient Fractionation**—Sucrose density gradient fractionation was performed essentially as described by Kippenberger *et al.* (5). 90% confluent cultures of PC12 cells from 2 10-cm plates were combined and homogenized in 1.4 ml of homogenization buffer (0.32 M sucrose, 10 mM HEPES, pH 7.1, 0.5 mM EDTA, protease inhibitors (Roche Applied Science)) using a ball-bearing cell cracker (17 passes, clearance 0.0007 in). Lysates were cleared of nuclei and unbroken cells with low speed spins (2  $\times$  10 min at 2010  $\times g$ ), and 500  $\mu$ l was layered over a 15-ml linear gradient of 0.6–1.6 M sucrose buffered with 10 mM HEPES, pH 7.1. Gradients were then centrifuged at 99,000  $\times g$  for 17–19 h at 4 °C (Beckman SW 40Ti rotor). One-milliliter fractions were collected from the top of tube.

#### RESULTS AND DISCUSSION

A high complexity yeast two-hybrid screen for Ral23V-interacting proteins resulted in the isolation of the previously characterized Ral-interacting proteins RalBP1/RLIP76 (18, 19) and Sec5 (1, 17) and revealed a novel candidate Ral effector, Exo84 (not shown). Iterative pairwise analysis revealed a specific interaction between human Exo84 and the human Ral GTPases (RalA and RalB) (Fig. 1A). Exo84 did not interact with any other tested GTPase (H-Ras, K-Ras, Rap1, Rap2, Arf1, Arf6, Rac1, Cdc42, Rab5, or Rab7), and Ral did not display an interaction with any other tested exocyst component other than Sec5 (human Sec3, Sec6, Sec8, Sec10, Sec15, and Exo70 were tested; not shown). Truncation analysis identified an amino-terminal domain (amino acids 122–333) as sufficient to interact with Ral in yeast (Fig. 1B). This domain completely overlaps with a predicted PH domain (SMART/PFAM) (Fig. 1C). As has been shown for the yeast orthologs, Exo84 associates with Sec5, a component of the exocyst we have previously defined as a Ral effector protein. As shown in Fig. 1B, the domain in Exo84 mediating interaction with Sec5 is non-overlapping with the Ral binding domain. *In vitro* binding assays using purified



**FIG. 1. Isolation of Exo84 as a candidate Ral target protein.** A, RalA or H-Ras were expressed in the L40 two-hybrid reporter strain as fusions to the LexA DNA binding domain together with Exo84 expressed as a fusion to the Gal4 transcriptional activation domain. Growth on media lacking leucine and tryptophan (*DO-LW*) indicates presence of plasmids expressing both hybrids. Growth on media lacking histidine (*DO-LWH*) indicates a positive two-hybrid interaction. *WT*, wild type. B, the indicated fragments of Exo84 were tested for interaction with Ral *versus* Sec5. C, predicted domain architecture of Exo84. The empirically defined Ral binding domain (*RalBD*) is indicated by the *black bar*. PFAM algorithms identify a predicted PH domain and a conserved domain from the Dor1-like family (*Dor1*) (27). Dor1 is involved with vesicle targeting to the Golgi in yeast. Scansite algorithms identify a putative PDZ domain ligand in the carboxyl-terminal tail.

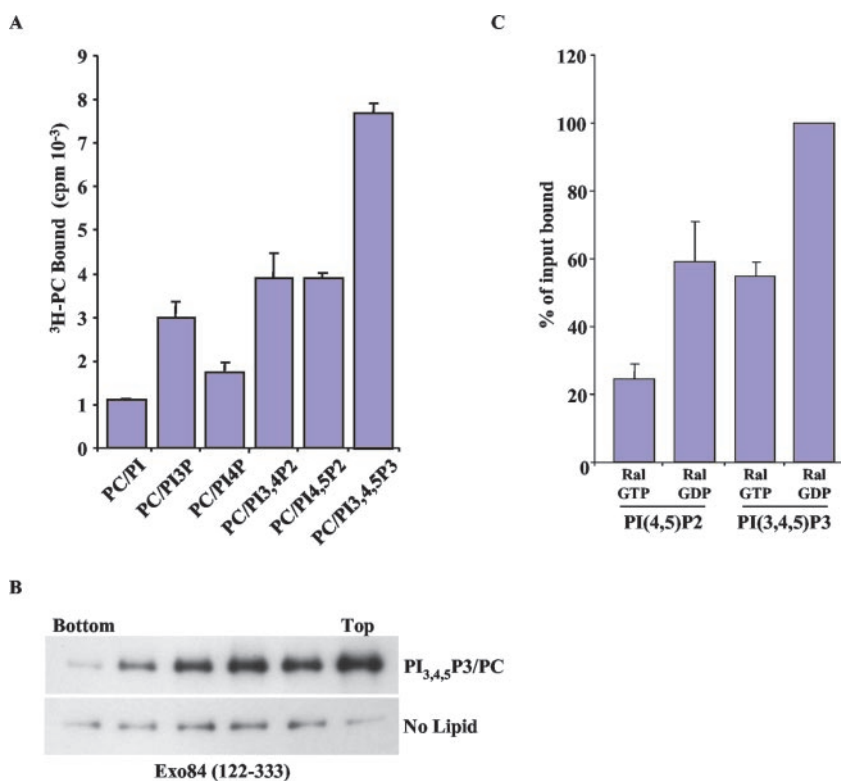
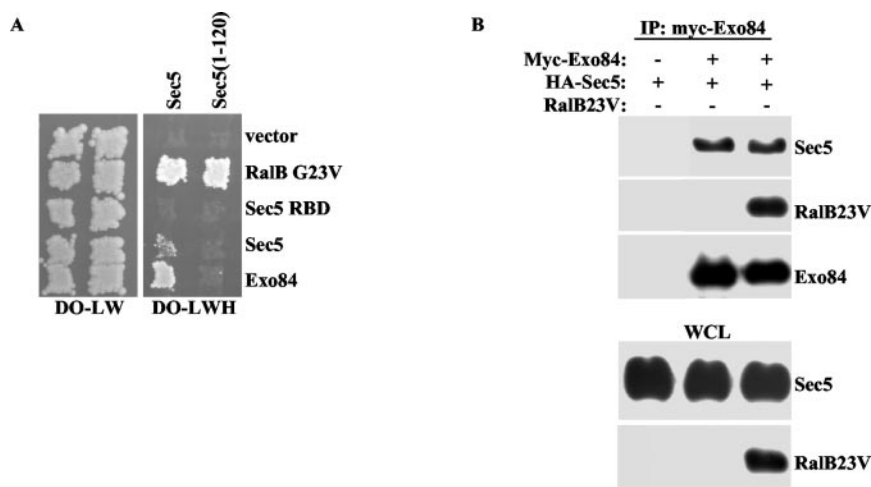


**FIG. 2. Exo84 interacts with activated Ral GTPases.** A, The Ral binding domain of Exo84 as defined in yeast (amino acids 122–333) or full-length Exo84 were expressed and purified from *Escherichia coli* as GST fusions. RalB was expressed and purified from *E. coli* as a poly-His<sub>6</sub> fusion. Recombinant Ral protein was loaded *in vitro* with GDP or GTPγS and incubated with the indicated immobilized GST fusions. After extensive washing, complexes were analyzed by immunoblotting with anti-RalB and anti-GST. B, ectopically expressed Myc-tagged RalB23V was immunoprecipitated (*IP*) with the 9E10 anti-myc antibody. The immunoprecipitates and whole cell lysates (*WCL*) were probed for the presence of Exo84 using a polyclonal anti-Exo84 antibody. C, 293T cells were transfected with the indicated untagged Ral variants together with Myc-tagged Exo84. Anti-Myc immunoprecipitates were probed for the presence of Ral and Exo84. D, PC12 cells were transfected with an Myc-tagged Ral binding domain of Exo84 (Exo84 (122–333)) alone or together with RalΔCAAX. Cells were stimulated or not with KCl. Representative confocal images of anti-Myc immunofluorescence are shown.

recombinant RalA and purified recombinant full-length Exo84 or the Ral binding domain of Exo84, Exo84-(122–333), established that RalA-GTP but not RalA-GDP can interact directly with Exo84 (Fig. 2A). In addition, activated Ral can associate with endogenous Exo84 in mammalian cells (Fig. 2B). Coimmunoprecipitation analysis using Ral effector domain variants reveals that the Ral/Exo84 interaction requires an intact Ral

effector domain and reveals a pattern of mutation sensitivity identical to that previously observed with Ral/Sec5 interactions (Fig. 2C) (1). KCl-induced membrane depolarization results in RalA activation in PC12 cells (1). Consistent with an interaction between Exo84 and activated Ral in cells, membrane depolarization resulted in recruitment of the minimal Ral binding domain to membrane-proximal regions, and this was blocked

**FIG. 3. Non-overlapping interaction domains mediate Exo84Sec5-Ral complex formation.** *A*, full-length Sec5 and the amino-terminal Ral binding domain of Sec5 were expressed as Gal4 fusions and tested for association with the indicated LexA DNA binding domain fusions. *DO-LW*, media lacking leucine and tryptophan; *DO-LWH*, media lacking histidine. *B*, hemagglutinin (*HA*)-tagged Sec5 was expressed in combination with RalB23V and Myc-Exo84 as indicated. Anti-Myc immunoprecipitates (*IP*) and whole cell lysates (*WCL*) were probed for the presence of the indicated proteins.



**FIG. 4. The Exo84 Ral binding domain overlaps a functional pleckstrin homology domain.** *A*, radiolabeled liposomes were prepared (3) using phosphatidylserine, [<sup>3</sup>H]phosphatidylcholine (*PC*), *PI*, and the indicated additional phosphoinositides. Raw counts per min (*cpm*) precipitated with GST-Exo84 (125–330) glutathione agarose are shown from triplicate analysis. *PI3P*, phosphatidylinositol 3-phosphate; *PI4P*, phosphatidylinositol 4-phosphate; *PI3,4P2*, phosphatidylinositol 3,4-bisphosphate; *PI4,5P2*, phosphatidylinositol 4,5-bisphosphate; *PI3,4,5P3*, phosphatidylinositol 3,4,5-trisphosphate. *B*, liposomes prepared as in *A* and rehydrated in 50% Nycodenz were mixed with GST-Exo84 (125–333) and layered under a 45, 40, 0% Nycodenz step gradient. Fractions were collected from the top after a 2-h spin at 70,000 rpm in a TLA100 rotor and probed for the presence of GST-Exo84. *C*, Exo84-mediated precipitation of radiolabeled liposomes was performed as in *A* after preincubation of Exo84 with Ral-GTP or RalGDP as indicated. % *cpm* bound was normalized to that obtained upon preincubation with RalGDP (arbitrarily set at 100). RalGDP had no effect on Exo84 association with liposomes (not shown).

by co-expression of a cytosolic activated Ral variant lacking carboxyl-terminal prenylation (Fig. 2*D*). These observations demonstrate that Ral forms activation-dependent complexes with two distinct subunits of the exocyst.

We have previously established that amino acids 1–120 of Sec5 are sufficient to interact with Ral-GTP *in vitro* and in cells (1). As shown in Fig. 3*A*, an adjacent non-overlapping region of Sec5 (amino acids 120–333) is sufficient to interact with Exo84. Together with the observation of non-overlapping Ral binding domains and Sec5 binding domains in Exo84, this suggests that Sec5 and Exo84 may have the capacity to independently associate with each other and with activated Ral proteins. Consistent with this, we see no inhibition of the formation of Exo84/Sec5 complexes in cells upon expression of activated RalA (Fig. 3*B*) despite the presence of significant amounts of RalA in the immunoprecipitates. Therefore, Ral activation likely does not restrict Exo84/Sec5 association. Rather, it may facilitate native complex assembly through stimulus-dependent recruitment of the Sec5 and Exo84 subunits.

As described above, SMART (20) and PFAM (21) algorithms predict a PH domain in Exo84 located in the middle of the empirically defined Ral binding domain (122–333). PH domains can associate with phosphatidylinositides and have been implicated in regulating recruitment of proteins to membranes and in regulating conformational changes within molecules containing PH domains that can modulate activity (22–24). The presence of a *bona fide* PH domain in Exo84 could contribute to compartmentalization of the protein and/or to regulation of assembly of Exo84 into an exocyst complex. We first tested the capacity of the predicted PH domain in Exo84 to interact with phospholipids *in vitro*. Radiolabeled liposomes (20  $\mu$ Ci of L-3-phosphatidyl[*N-methyl*-<sup>3</sup>H]choline,12-dipalmitoyl) containing either phosphatidylinositol 4,5-bisphosphate or PI3,4,5P3 could be precipitated with recombinant GST-Exo84-(122–333) glutathione-agarose (Fig. 4*A*) with a small preference for PI3,4,5P3. In addition, incubation of Exo84-(122–333) with PI3,4,5P3-containing liposomes allowed a significant fraction of the Exo84 to float up a Nycodenz density gradient (Fig. 4*B*,

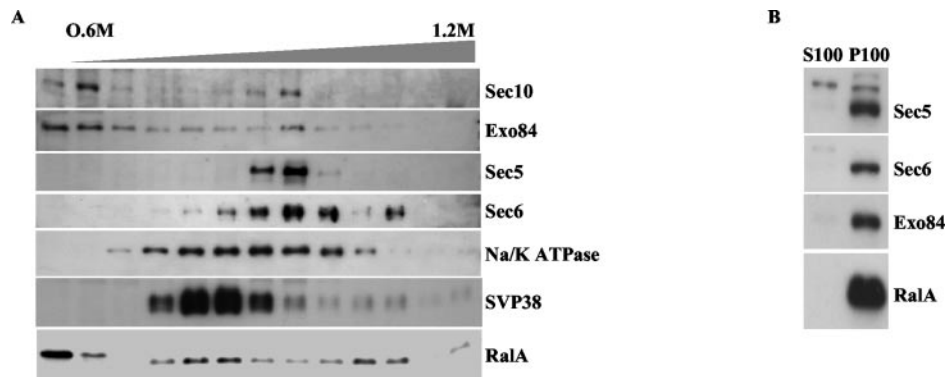


FIG. 5. **Distinct density gradient distribution of Exo84 versus Sec5.** A, mechanically disrupted PC12 cell lysates (cleared of nuclei and unbroken cells) in 0.3 M sucrose were layered on top of a linear 0.6–1.6 M sucrose gradient. After a 19-h spin at 99,000 G (SW 40Ti rotor) fractions were collected from the top, separated by SDS-PAGE, and blotted with the indicated antibodies. B, PC12 cell lysates prepared as in A were centrifuged at  $100,000 \times g$  for 45 min. Cell equivalent supernatant (S100) and pellet (P100) fractions were probed for the presence of the indicated proteins.

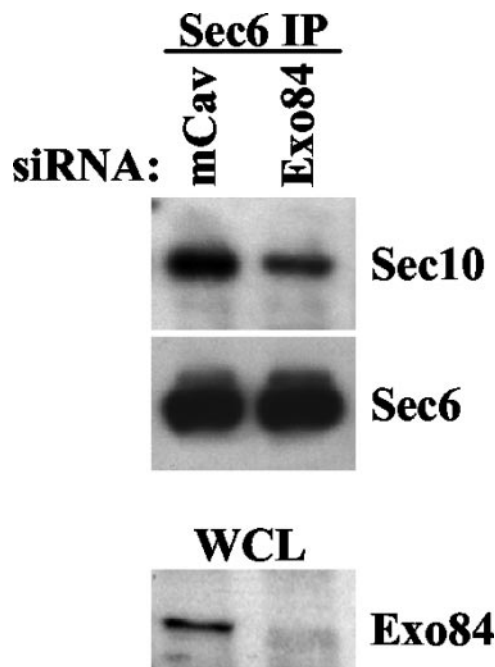


FIG. 6. **Exo84-dependent assembly of Sec6Sec10 complexes.** HeLa cells were transfected with siRNAs targeting human Exo84 or mouse caveolin1 (*mCav*). 72 h post-transfection cells were harvested, and endogenous Sec6 was immunoprecipitated from non-denaturing lysates. The immunoprecipitates were probed for the presence of Sec6 and Sec10. Whole cell lysates (WCL) were probed with anti-Exo84 to monitor effectiveness of siRNA treatment.

compare PI liposomes (*bottom panel*) to PI3,4,5P3 liposomes (*top panel*). Because the PH domain of Exo84 overlaps with the Ral binding domain, we examined the consequences of recombinant Ral-GTP and Ral-GDP on *in vitro* association of Exo84-(122–333) with phosphoinositides. As shown in Fig. 4C, Ral-GTP can compete with PI3,4,5P3-containing liposomes for association with Exo84. As far as we are aware, this is the first observation of an activation-dependent association of a small G-protein with a PH domain and may represent a novel mechanism for GTPase-dependent regulation of protein/lipid interactions.

Work in yeast suggests that the exocyst may be a dynamic complex, with subsets of components forming both a targeting patch on the plasma membrane and a subcomplex on secretory vesicles (10, 13, 25). Analysis of protein interactions and subunit compartmentalization suggests that yeast Sec 15, Sec10, and Exo84 are on a vesicle-associated subcomplex that may

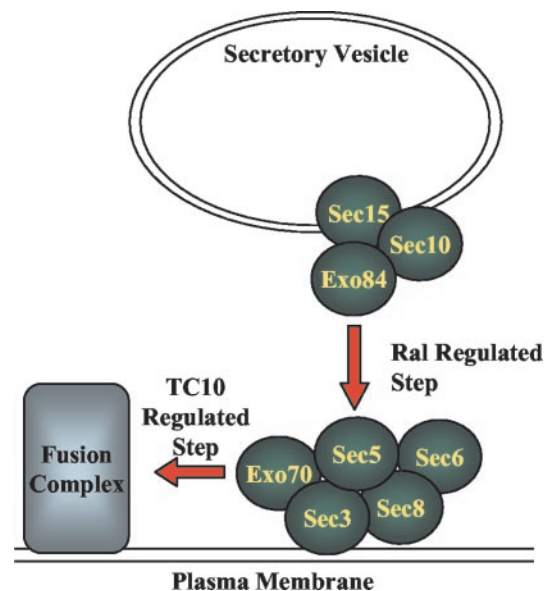


FIG. 7. **Ral GTPases regulate an exocyst subcomplex assembly interface through dual effector interactions.**

function as a bridge between secretory vesicles and their sites of fusion (13, 25). In yeast, formation of this bridge appears to be regulated by the Sec4 GTPase (13). Loss of RalA expression in human cells inhibits assembly of Sec10 into a complex with Sec6 but does not affect plasma membrane compartmentalization of Sec6 (1). This together with the observation that Ral GTPases can interact with two different components of the exocyst (Sec5 and Exo84) suggests that, like yeast, the human exocyst may be a dynamic complex, the assembly of which is regulated by Ral GTPases. To begin to address the possibility that components of the mammalian exocyst may exist in subcomplexes before secretory vesicle targeting, we examined the relative distribution of endogenous exocyst components on linear 0.6–1.2 M sucrose gradients. We chose to examine PC12 cells given our earlier observations implicating Ral in the regulation of stimulus-dependent exocytosis (1). Fractions from detergent-free mechanical lysates were blotted with synaptophysin as a secretory/dense core vesicle marker and (Na<sup>+</sup>K<sup>+</sup>)-ATPase as a plasma membrane marker and with the indicated exocyst subunits. As shown in Fig. 5A, the Sec5 signal peaks together with Sec6, although Sec5 shows a tighter distribution on the gradient. In contrast, the majority of Exo84 is in the top half of the gradient together with Sec10. This migration is most likely due to the association of Exo84 and Sec10 with a light



membrane fraction, because Exo84 sediments during differential centrifugation (Fig. 5B). RalA protein appears to be distributed across the gradient with two peaks, one of which co migrates with the secretory vesicle marker synaptophysin. A population of slower migrating RalA, which may lack prenyl group modification, remains in the top of the gradient together with Sec10 and Exo84. The distinct distribution of Exo84 relative to Sec5 suggests that, as in yeast, exocyst subunits exist in subcomplexes in cells. Cofractionation of Exo84 together with Sec10 *versus* cofractionation of Sec5 together with Sec6 places the two Ral effectors on each of the presumed exocyst subcomplexes, suggesting that a subpopulation of Ral may regulate the full complex assembly interface. Consistent with action of Exo84 at the octameric exocyst complex assembly interface, inhibition of native Exo84 expression reduced complex formation between Sec6 and Sec10 (Fig. 6).

In yeast, Exo84 is required for exocytosis and interacts with Sec10 and Sec5. Exo84 localization with sites of active exocytosis on the plasma membrane is Sec5-dependent. However, Exo84 association with Sec10 is not (25). Sec10 in turn is associated with Sec15, an exocyst subunit localized to secretory vesicles (13), whereas Sec5 is plasma membrane localized independently of Exo84 (25). As mentioned above these observations suggest that in yeast there may be a subcomplex of Exo84, Sec10, and Sec15 required to bridge secretory vesicles to an exocyst-targeting patch on the plasma membrane (that includes Sec5, Sec6, Sec8, Sec3, and Exo70) (10). Exo84 and Sec5 may function at the assembly interface, because overexpression of Exo84 can partially complement the secretion defect of a temperature-sensitive Sec5 mutant. The observations described in this study are consistent with a similar exocyst anatomy in mammalian cells and suggest a rough working hypothesis for the contribution of Ral GTPases to exocyst function; that is, that the dual association of Ral-GTP with Exo84 and Sec5 regulates the assembly of a full exocyst complex to tether secretory vesicles before docking and fusion (Fig. 7). Recent observations suggest that subsequent coupling of the exocyst to fusion machinery may be regulated by another small G-protein, TC10, through interaction with Exo70 (26). The

biophysical nature of dynamic Ral-dependent exocyst assembly remains to be determined. However, the observations described here will facilitate further studies directed at defining dynamic control of exocyst function and the contribution of Ral GTPases to regulated vesicle trafficking.

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**Ral GTPases Regulate Exocyst Assembly through Dual Subunit Interactions**  
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