Interactions between Rabs, tethers, SNAREs and their regulators in exocytosis

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Abstract
Sec2p is the exchange factor that activates Sec4p, the Rab GTPase controlling the final stage of the yeast exocytic pathway. Sec2p is recruited to secretory vesicles by Ypt32p-GTP, a Rab controlling exit from the Golgi. Sec15p, a subunit of the octameric exocyst tethering complex and an effector of Sec4p, binds to Sec2p on secretory vesicles, displacing Ypt32p. Sec2p mutants defective in the region 450–508 amino acids bind to Sec15p more tightly. In these mutants, Sec2p accumulates in the cytosol in a complex with the exocyst and is not recruited to vesicles by Ypt32p. Thus the region 450–508 amino acids negatively regulates the association of Sec2p with the exocyst, allowing it to recycle on to new vesicles. The structures of one nearly full-length exocyst subunit and three partial subunits have been determined and, despite very low sequence identity, all form rod-like structures built of helical bundles stacked end to end. These rods may bind to each other along their sides to form the assembled complex. While Sec15p binds Sec4-GTP on the vesicle, other subunits bind Rho GTPases on the plasma membrane, thus tethering vesicles to exocytic sites. Sec4-GTP also binds Sro7p, a yeast homologue of the Drosophila lgl (lethal giant larvae) tumour suppressor. Sro7 also binds to Sec9p, a SNAP25 (25 kDa synaptosome-associated protein)-like t-SNARE [target-membrane-associated soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor] (SNARE), and can form a Sec4p–Sro7p–Sec9p ternary complex. Overexpression of Sec4p, Sro7p or Sec1p (another SNARE regulator) can bypass deletions of three different exocyst subunits. Thus promoting SNARE function can compensate for tethering defects.

Introduction
Vesicular traffic is the predominant mechanism by which components are transported from one membrane-bounded organelle to another in eukaryotic cells. This process can be divided into several distinct steps: cargo selection, vesicle formation, vesicle delivery, tethering of the vesicle to the target membrane and fusion of the vesicle membrane with that of the target. Rab GTPases have been implicated in the regulation of each of these steps. We have been studying Sec4p, the Rab GTPase that controls the final stage of the exocytic pathway in the yeast Saccharomyces cerevisiae, as well as the proteins required to activate Sec4p and those that respond to activated Sec4p. These studies reveal a network of proteins that act to couple each stage of transport with the next along the pathway in a highly choreographed series of events.

Key words: exocytosis, guanine nucleotide-exchange factor, Rab GTPase, secretory vesicle, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE), tether. Abbreviations used: Cdc42, cell division cycle 42; GEF, guanine nucleotide-exchange factor; lgl, lethal giant larvae; (t-)SNARE, (target-membrane-associated) soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor.

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A Rab cascade
Activation of Sec4p requires dissociation of bound GDP by a GEF (guanine nucleotide-exchange factor) called Sec2p. Both Sec2p and Sec4p are concentrated on the surface of secretory vesicles as they are delivered to exocytic sites at the tip of the bud, early in the cell cycle, and at the neck separating the mother and daughter cells, as they approach cytokinesis [1]. The association of Sec2p with secretory vesicles requires a small region in the C-terminal half of Sec2p (amino acids 450–508) termed the localization domain, while the exchange activity is confined to the N-terminus (amino acids 1–160) [2]. To explore the mechanism by which Sec2p associates with vesicles, we screened for high copy number suppressors of a missense mutation, sec2-78, within the localization domain. sec2-78, within the localization domain. Overexpression of either Ypt31p or Ypt32p restores the localization of the Sec2-78p mutant [3]. Ypt31p and Ypt32p are redundant Rab GTPases homologous with mammalian Rab11 that regulate export from the Golgi apparatus, the step just prior to that controlled by Sec4p [4,5]. Biochemical studies demonstrated that Sec2p binds Ypt32p when it is in its GTP-bound form and that binding requires a region (amino acids 161–258) downstream of the exchange domain (amino acids 1–160) but upstream of that implicated in localization (amino acids 450–508) [3]. Thus Sec2p is both a GEF for one Rab and an effector for the Rab that acts just upstream. This defines a Rab cascade that may act to couple adjacent...
stages of membrane traffic. Subsequent studies have suggested that Rab cascades may be a recurring if not a general theme in membrane traffic [6,7].

A reversible interaction of Sec2p with the exocyst

The studies described above clearly implicated Ypt32p in the localization of Sec2p. However, they did not clarify the role of the localization domain of Sec2p, as it is not needed for Ypt32p binding [3]. The role of this region has recently been defined through our studies of the exocyst complex [12].

The exocyst is a large complex containing eight conserved gene products, each required for exocytosis [8,9]. Early genetic studies suggested that one subunit, Sec15p, might act downstream of Sec4p [10] and subsequent biochemical analysis confirmed that Sec15p is a bona fide Sec4p effector as it directly binds Sec4p-GTP [11]. In the small GTPase field, there are numerous examples of GEFs that bind to an effector of the GTPase that they activate. Such interactions can be used to increase signalling specificity or to establish a positive-feedback loop that maintains a localized domain of activated GTPase. We explored the possibility that Sec15p, a Sec4 effector, might bind Sec2p, the Sec4p GEF [12]. Both co-precipitation studies and binding studies with purified recombinant proteins demonstrated specific binding of Sec15p to Sec2p. While using Sec2p truncation constructs to define the Sec15p-binding site, we made a very interesting observation. Full-length Sec2p co-precipitated approx. 2% of the Sec15p in a lysate, while Sec2p truncations missing the C-terminal half of the protein bound 15-fold more efficiently. The region of Sec2p involved in restricting Sec15p binding, amino acids 450–508, is precisely the same region needed for localization. Furthermore, the sec2-78 missense mutation (C483Y) lies within this region and both prevents proper localization and allows strong binding to Sec15p [12].

Fractionation studies established that full-length Sec2p interacts with Sec15p only on membranes, most likely secretory vesicles, even though the bulk of both Sec2p and Sec15p are found in the cytosol. In contrast, truncated alleles of Sec2p bind Sec15p more efficiently and predominantly in the cytosolic fraction [12]. This suggests that the interaction of Sec2p with Sec15p is normally restricted to the membrane fraction by a mechanism requiring the localization domain. In the absence of this regulation, Sec2p is constitutively bound by Sec15p and is unavailable for binding to new secretory vesicles. Hence activation of Sec4p fails.

The actual binding site for Sec15p, amino acids 161–258, could not be resolved from the Ypt32p-binding site. Sec15p and Ypt32p compete with each other for binding to Sec2p, and the affinity of the Sec2p–Sec15p interaction is approx. 3-fold higher than the Sec2p–Ypt32p interaction [12]. Thus overproduction of Ypt32p is able to suppress the localization defect of the Sec2p mutants because at high levels it can displace the bound Sec15p.

In total, our results suggest the following scenario (see Figure 1): Sec2p is recruited to secretory vesicles by Ypt32-GTP. Sec2p then activates Sec4p, which in turn recruits its effector, Sec15p. Sec15p binds to Sec2p, displacing Ypt32p. The interaction of Sec2p with Sec15p helps to establish and maintain a domain of highly activated Sec4p on the vesicle. After the vesicle is tethered at the plasma membrane, the localization domain is used to displace Sec2p from Sec15p. Sec2p thus becomes available to bind a new vesicle. While the signal that triggers displacement of Sec15p is not yet known, the localization domain of Sec2p is highly phosphorylated, suggesting the involvement of a kinase [2].

Structure and function of the exocyst

Phenotypic analysis of exocyst mutants indicates that the complex functions after vesicles have been delivered to exocytic sites, but prior to the formation of SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) complexes [1,13]. Six of the eight exocyst subunits (Sec5p, Sec6p, Sec8p, Sec10p, Sec15p and Exo84p) are delivered to exocytic sites on secretory vesicles [14], while Sec3p appears to use a vesicle-independent mechanism [15] that relies on a direct interaction with either Cdc42 (cell division cycle 42)-GTP [16] or Rho1-GTP [17] to define these sites (see Figure 2). Exo70p can use both vesicle-dependent and vesicle-independent mechanisms [14] and directly binds Rho3-GTP [18,19]. Our working model has been that the exocyst complex is only fully assembled once the vesicles arrive at the sites marked by Sec3p and Exo70p. Sec15p directly interacts with the polarity marker Bem1p [20,21] and this interaction is also important in defining the location of exocytic sites. While the mammalian exocyst complex interacts with a somewhat different set of proteins, the underlying mechanism is thought to be the same; different subunits interact with different components on either the vesicle or the plasma membrane and the exocyst integrates the different signals to determine where and when vesicles are tethered in preparation for fusion.
Figure 2 | Model of exocyst assembly and structure
The exocyst complex is assembled from eight subunits, of which at least four are composed, in part or in whole, of helical bundles arranged end to end into long rods. These rods align in a side-to-side fashion in the fully assembled complex. Six of the subunits ride the vesicle along actin cables to exocytic sites, while Sec3p and Exo70p can recognize these sites by an actin-independent mechanism. Sec3p binds Rho1-GTP or Cdc42-GTP, while Exo70p binds Rho3-GTP and Sec15p binds Sec4-GTP.

This past year has seen major advances in our knowledge of the structure of the exocyst. The crystal structures of nearly full-length Exo70p [19,22] and the C-terminal third of Exo84p [19], Sec15p [23] and Sec6p [24] have been solved. Although these different subunits show very little sequence similarity, the structures show a strikingly similar architecture. In each case, helical bundles are arranged end to end to form long rod-like structures. In the case of Exo70p, the rod is 3 nm wide by 16 nm in length. A prior electron microscopy study provided low-resolution images of the mammalian exocyst either unfixed or pre-fixed in glutaraldehyde [25]. The unfixed complexes appear as flowers with four to six rod-shaped ‘petals’ each with dimensions (4–6 × 10–30 nm) roughly comparable with those established for Exo70p. In the pre-fixed preparation, the ‘petals’ appear to be aligned in a side-to-side fashion to yield one large, elongated structure of larger width (13 nm × 30 nm). It is interesting to speculate that the apparent opening and closing of this ‘flower’ may reflect a normal function of the exocyst (see Figure 2) in which it initially tethers a vesicle to the plasma membrane at a considerable distance in its open conformation, but as the subunits align alongside one another the vesicle is drawn close enough to the plasma membrane to promote SNARE complex formation and fusion.

Sro7p links Sec4-GTP to a t-SNARE
In a search for novel Sec4p effectors using an affinity purification method, we identified Sro7p. Binding studies with purified proteins confirmed that Sro7p directly and specifically binds Sec4-GTP [26]. Sro7p is a member of the Igl (lethal giant larvae) protein family that has been implicated in cell polarity in animal epithelial cells. In Drosophila, Igl was first identified as a tumour suppressor. The excess cell growth in Igl mutant larvae may result from the loss of cell polarity [27]. In yeast, Sro7p is important for exocytosis and has been shown to bind to Sec9p, the yeast homologue of the SNAP25 (25 kDa synaptosome-associated protein) t-SNARE [28]. We found that a ternary complex could be formed using purified recombinant proteins in which Sro7p coupled Sec4-GTP to Sec9p [26]. Thus Sro7p acts as a link between an activated Rab protein and a t-SNARE. Sro7 may act to promote SNARE complex formation or SNARE-mediated fusion in response to Sec4-GTP.

Genetic interactions between the exocyst and SNARE regulators
All subunits of the exocyst are essential for growth except for Sec3p [29]. Cells lacking Sec3p are able to grow at low temperature on synthetic media, but growth and secretion fail at elevated temperatures. The level of assembled exocyst complex is greatly reduced in sec3Δ cells [29]. We found that overproduction of Sec4p, Sro7p or Sec1p was able to suppress the growth defects of a sec3Δ mutant, but exocyst assembly was not restored. Most strikingly, overexpression of Sec4p, Sro7p or Sec1p was able to bypass the otherwise lethal deletions of sec5 and exo70 [30]. Suppression by Sec4p required Sro7p function, while suppression by overexpression of Sec1p did not require Sro7p. Sec1p binds to assembled exocytic SNARE complexes and is essential for fusion [31].

We offer the following model to account for these observations (see Figure 3): Under normal circumstances, the exocyst holds vesicles in proximity to the plasma membrane long enough for SNARE complexes to form and mediate fusion in response to Sec4p, Sro7p and Sec1p. When a subunit of the exocyst is missing, the resulting exocyst complexes are unstable and vesicles are lost from exocytic sites before SNARE complexes can be assembled and function. However, when Sec4p, Sro7p or Sec1p is overexpressed, the unstable
Figure 3 | Model for the Sec4p signalling pathways
Secretory vesicles (V) carry the Rab GTPase Sec4p and its GEF Sec2p, which keeps Sec4p in its activated, GTP-bound state. Sec15p, a member of the exocyst complex, is one effector for Sec4p, and the interaction of these two proteins is required for the assembly of this complex and its tethering function in exocytosis. Sec1p binds to assembled SNARE complexes, possibly stabilizing them. Another effector of Sec4p, Sro7p, interacts with the exocytic t-SNARE Sec9p and this interaction is required for Sec4p’s role in exocytosis. Arrows indicate physical interactions.

Tethering by the incomplete exocyst complexes is adequate because SNARE function is enhanced. Sec4p acts upstream of Sro7p and therefore requires it for suppression, while Sec1p acts either downstream of Sro7p or independent of it, and thus suppression by Sec1p does not require Sro7p. Further analysis will be needed to explore this model.

References

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