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Rabs and EHDs: alternate modes for traffic control

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Synopsis

Endocytic trafficking is a highly organized process regulated by a network of proteins, including the Rab family of small GTP-binding proteins and the C-terminal EHDs (Eps15 homology-domain-containing proteins). Central roles for Rab proteins have been described in vesicle budding, delivery, tethering and fusion, whereas little is known about the functions of EHDs in membrane transport. Common effectors for these two protein families have been identified, and they facilitate regulation of sequential steps in transport. By comparing and contrasting key aspects in their modes of function, we shall promote a better understanding of how Rab proteins and EHDs regulate endocytic trafficking.

Keywords

collapsin response mediator protein-2 (CRMP2); endocytic trafficking; Eps15 homology (EH); Eps15 homology-domain-containing protein (EHD); GTPase-activating protein (GAP); Rab; transferrin receptor (TfR)

INTRODUCTION

Endocytic trafficking is a basic requirement for all cells, as it is essential for the internalization and transport of nutrients, segments of plasma membrane and cell surface receptors [1–4]. It is a highly organized process regulated by a network of proteins, among which are the Rab family of small GTP-binding proteins and the EHDs (Eps15 homology-domain-containing proteins).

Rab proteins constitute the largest of the Ras superfamily of small GTP-binding proteins. There are 11 Rab members in budding yeast, termed Ypts, and more than 60 members have been identified in humans. Besides their central role in membrane trafficking, Rabs and their effector proteins are also implicated in various processes including cell signalling [5,6], cell migration [7], cytokinesis [8,9], ciliogenesis [10–12], apoptosis [13,14] and autophagy [15–18]. Dysfunction of Rab proteins is associated with cancer, pathogen-related diseases and inherited disorders [19].

The human EHD family was initially characterized about a decade ago (reviewed in [20,21]). This family consists of four highly homologous mammalian members, EHD1–4.

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The single orthologue of human EHD1 in *Caenorhabditis elegans* is known as Rme-1 [22], but there are no real homologues in *Saccharomyces cerevisiae*. All four mammalian EHDs are involved in the regulation of endocytic transport, albeit at distinct but partially overlapping steps [20,23]. EHDs exert many of their regulatory functions through various binding partners. The majority of these interactions are through the C-terminal EH domain of the EHDs, which bind to an NPF (asparagine-proline-phenylalanine) motif present in EHD-binding proteins [20,23]. Although the aberrant expression of EHDs has been documented in various diseases, it remains unclear as to whether this altered expression has any physiological significance.

Interestingly, some EHD-binding partners are also Rab effectors. To date, however, it remains unclear as to whether they coordinately control transport or merely take advantage of the same effectors. In the present review, we shall compare key aspects of the EHD and Rab family proteins and provide a better understanding of how they each control endocytic transport.

FUNCTIONS IN ENDOCYTIC TRAFFICKING

Rab GTP-binding proteins, together with their diverse effectors, are implicated in all steps of endocytic transport, including vesicle formation, motility, tethering/docking and fusion [19,24]. A classic example of Rab protein involvement in vesicle formation is provided by Rab5. The Rab5–GDI (GDP dissociation inhibitor) complex is required for clathrin-coated vesicle formation *in vitro* [25] and overexpression of a Rab5-GAP (GTPase-activating protein), RN-Tre, inhibits epidermal growth factor receptor internalization [26]. Other examples of Rab involvement in vesicle budding include Rab4 and Rab7; an *in vitro* assay indicates that vesicle formation is regulated by Rab4 and its effector, rabaptin-5/rabex-5 [27], whereas Rab7 is involved in macropinosome formation [28]. Rab regulation of vesicle transport along the actin filaments and microtubules is through direct or indirect interaction with motor proteins, which will be discussed subsequently in this review.

Among the most widely recognized roles for Rabs is the tethering and fusion of vesicles to target organelles. Rabs recruit tethering factors that form bridges between vesicles and target membranes, which in turn lead to membrane fusion through Rab effector–SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) interactions [19,24,29]. For example, Rab5 serves as a regulator of early endosomal tethering and fusion. The Rab5 effector EEA1 (early endosome antigen 1) bridges Rab5-positive vesicles through two Rab5-binding sites on both the N- and C-termini [30], and subsequently mediates membrane fusion by direct interactions with the SNARE proteins syntaxin 6 and syntaxin 13 [31,32].

Although Rabs are primarily involved in vesicle tethering and fusion, EHDs may function as ‘pinchases’ in vesicle scission, thus regulating vesicle transport from endosomes to downstream compartments [33]. Despite a high level of sequence identity between the four paralogues (67.9–86.5%), EHDs regulate different steps of endocytic traffic. However, loss of expression of one EHD can apparently be partially compensated by other EHDs ([34–36], and reviewed in [23]). EHD1 is the most widely studied family member, and its best-

characterized role is in regulating the recycling of membrane cargo from the ERC (endocytic recycling compartment) to the plasma membrane. EHD1 regulates both clathrin-dependent and -independent pathways, including TfR (transferrin receptor) [37], MHCI [38] and β1 integrins [39]. On the other hand, the precise function of EHD2 is yet to be elucidated. It is involved in the internalization of TfR and the glucose transporter GLUT4 [40] and displays functional redundancy with EHD1 in some tissues [35]. EHD3 is the closest paralogue to EHD1, sharing 86% amino acid identity and it regulates retrograde transport from the EE (early endosome) to the Golgi [41,42]. EHD4 negatively regulates Rab5 and has been implicated in vesicle transport from the EE to the ERC, as well as from the EE to the lysosomal degradation pathway [35,43].

Choose your nucleotide

Rab proteins function as molecular switches between active GTP-bound forms and inactive GDP-bound forms. GDP-bound Rab is stabilized in its inactive cytosolic form by GDI [44–46], and its targeting to specific membranes is mediated by the membrane-bound GDFs (GDI displacement factors) [47]. Subsequently, GEFs (guanine-nucleotide-exchange factors) catalyse the exchange of GDP for GTP and convert Rabs into membrane-bound active forms that are recognized by various effector proteins. Finally, GAPs stimulate the hydrolysis of GTP, and convert Rabs back into the GDP-bound inactive form [48,49].

Surprisingly, despite the similarity of the EHD nucleotide-binding G-domain to those found in Ras and dynamin family GTPases, EHDs display a much higher affinity for ATP than GTP. Nonetheless, the rate of ATP hydrolysis stimulated by EHDs is 600-fold slower than GTP hydrolysis by dynamin [33]. The structure of EHDs consists of two helical domains with a nucleotide-binding domain in between, a linker region and a C-terminal EH domain [33] (Figure 1). Cytoplasmic-localized EHDs bind ATP and oligomerize along tubular membranes. Unlike Rab proteins, which depend on extrinsic GTPase activity via GAPs, EHDs have intrinsic ATPase activity that is stimulated by membrane binding. ATP hydrolysis induces a conformational change that destabilizes the membrane, which in turn leads to vesicle scission [33].

Hanging on

Rab GTPases are reversibly associated with membranes by prenylation, whereas membrane binding of EHDs is mediated by their lipid-binding helical regions [33] or through direct binding of C-terminal EH domains to phosphatidylinositols [50]. In addition, EH domain binding to an array of NPF-containing interaction partners might facilitate either recruitment of EHD to membranes or stabilization on the membranes [51]. Cytosolic GDP-bound Rabs are recognized by REP (Rab escort protein), which presents Rabs to GGT (geranylgeranyltransferase) [52]. Rab proteins are then prenylated on one or two C-terminal cysteine residues by GGT and the prenylated Rabs are bound to GDIs. The GDI–Rab complex is recognized by a membrane-bound GDF, which promotes the release of the GDI [47]. After fulfilling their various functions at the membrane through the GTP–GDP cycle regulated by GEFs and GAPs, Rab proteins return to the inactive GDP-bound form and then can be extracted from the membrane by GDIs [44–46,53,54].

Compared with the membrane-association cycle of Rab proteins controlled by Rab regulatory proteins, the mechanism of the EHD interaction with membranes remains to be elucidated. Although EHDs are capable of tubulating liposomes *in vitro* [33], their interaction with membranes *in vivo* may be more complex. It has been shown that depletion of EHD1 has no effect on the tubular membrane localization of the double-palmitoylated and -farnesylated C-terminus of H-Ras [55]. Furthermore, MICAL-L1 (molecule interacting with CAsL-like 1), an EHD1-binding partner, recruits and/or stabilizes EHD1 on tubular membranes [51]. These results suggest that EHDs associate with pre-existing tubular membranes *in vivo*, and may exert their roles in membrane tubulation co-operatively with their diverse binding partners, including the BAR (Bin/amphiphysin/Rvs)-domain-containing protein AMPH-1/amphiphysin/Bin1 [56]. Accordingly, EHDs may behave similarly to dynamin, which tubulates membranes *in vitro* [57] and *in vivo* [58], whereas its main role is to form a spiral at the neck of budding vesicles and stimulate membrane scission [59–62].

EHDs against Rabs: united or alone

While Rabs function as monomers, oligomerization is required for EHD ATPases to function. Dimerization of EHDs is mediated by a hydrophobic interface in the nucleotide-binding G domain [33]. Where this dimerization occurs is not clear. One possibility is that it occurs in the cytoplasm and is required for membrane association. A different possibility is that dimerization might ensue on interaction with the membrane to stabilize binding. Indeed, EHD mutants impaired in their ability to homo- or hetero-oligomerize display a cytoplasmic distribution [42], suggesting that oligomerization is required for association or continued contact with tubular membranes. Subsequently, it has been predicted that the EHD ring structures which are generated as a result of advanced oligomerization stimulate ATP hydrolysis, thus leading to membrane fission and the release of vesicles [33].

Membrane bending activity by EHDs

Similar to the dynamin superfamily of GTPases, EHD2 is capable of tubulating liposomes *in vitro* in a nucleotide-independent manner [33]. Moreover, it has been predicted that EHD2 oligomerizes in ring-like structures around lipid tubules, and that the membrane curvature imposed by the proposed oligomer is perpendicular to the curved membrane interface of the EHD2 dimer [33]. Mathematical modelling of EHD2 ring-like structures along the membrane tube suggests that EHD2 induces membrane bending through a scaffolding mechanism that can be described by the Helfrich model of membrane elasticity [63]. This model formulates the membrane curvature as a mathematical surface so that the energy needed for membrane fission can be calculated [63,64]. The model also contends that additional protein players may be required to reduce the energy barrier and facilitate fission of the membrane [63]. To date, there is no membrane bending function reported for Rab proteins.

Interaction with motors

The movement of vesicles along actin microfilaments or microtubules is propelled by motor proteins, which include the actin-dependent motors of the myosin family and the microtubule-dependent motors: kinesin superfamily proteins and cytoplasmic dynein.

Rabs are involved in regulating all three types of motors [19,29] (Figure 2B). One example of a Rab interaction with actin motors is via the Rab effector protein, Rab11-FIP2 (Rab11-family interacting protein 2), which is also an EHD-binding partner [42]. Rab11-FIP2 connects Rab11 and myosin Vb and regulates vesicle recycling [65]. Another example is the recent discovery that Rab6 controls the fission of Rab6 vesicles from the Golgi complex through its interaction with myosin II [66].

Kinesins can be directly or indirectly regulated by Rabs. Direct interaction between Rab6 and the kinesin-like protein Rabkinesin 6 is essential for cytokinesis [67,68]. An example of indirect regulation is the kinesin KIF16B (kinesin 16B) that is localized to phosphatidylinositol-3-phosphate-positive endosomal membranes and controlled by Rab5 [69].

Indirect interactions between dynein and Rabs have also been described. RILP (Rab7-interacting lysosomal protein) binds to active Rab7 and mediates minus-end-directed transport of late endosomes by recruiting the dynein–dynactin complex [70]. A new study has shown that the Rab11 effector protein Rab11-FIP3 interacts with the DLIC-1 (dynein light intermediate chain 1) and mediates transport from sorting endosomes to the ERC [71].

Compared with Rab proteins, little is known about the interactions between EHDs and motor proteins. A recent study has shown that CRMP2 (collapsin response mediator protein-2), a novel binding partner of the EHD1-interaction protein MICAL-L1, serves as a link between MICAL-L1-EHD1-associated endosomes and cytoplasmic dynein [72] (Figure 2A). Another report demonstrated that myosin Vb co-localizes with Rab8a on tubules containing EHD1 and EHD3 [73], suggesting that EHDs and Rab proteins may function coordinately in regulating vesicle transport through their interactions with common motor proteins. Further investigation will be required to better understand the mechanism by which EHDs interact with motor proteins and the regulatory effect on vesicle transport.

Effectors and binding partners

Rab effectors are proteins that generally bind to a specific GTP-bound Rab and mediate at least one set of downstream effects. On the other hand, EHD-binding partners are proteins that usually contain an NPF motif flanked by acidic residues [20,23,74]. Both Rab proteins and EHDs appear to have multiple effectors or binding partners through which diverse functions of membrane transport are facilitated. For example, an affinity chromatography approach yielded 22 proteins that potentially interact with Rab5-GTP [75]. A wide range of Rab- and EHD-binding partners have been summarized in several reviews [20,23,29].

The various Rab effectors share little sequence homology, although the binding interfaces usually involve the two switch regions of Rab proteins [76]. Many Rab effectors contain more than one binding domain for distinct binding partners, suggesting these multivalent molecules may play roles in coordinating interrelated endocytic pathways [77,78]. On the contrary, EHD-binding partners almost always contain an NPF tripeptide sequence followed by acidic residues and interact with EHDs via NPF motif-EH domain binding. The highly positively charged surface of the EH domain dictates the requirement for flanking acidic residues [74,79].

So far, three proteins have been identified that serve both as Rab effectors and as EHD-binding partners: Rabenosyn-5, Rab11-FIP2 and MICAL-L1. The identification of these proteins raises the possibility that Rab proteins and EHDs might coordinately regulate intracellular trafficking.

Rabenosyn-5, a divalent effector of Rab4 and Rab5, binds to the EHD1 EH domain through the first two of its five NPF motifs [80]. The distribution patterns of internalized transferrin and MHCI in cells depleted of both Rabenosyn-5 and EHD1 is similar to cells depleted only of Rabenosyn-5, indicating that Rabenosyn-5 acts upstream of EHD1. These results suggest that Rabenosyn-5 may connect with Rab4 on early endosomes, and subsequently with EHD1 at the ERC, thus linking these two consecutive pathways to regulate cargo recycling to the plasma membrane.

Rab11-FIP2, the only known Rab11 effector that contains three NPF motifs, binds to the EHD1 and EHD3 EH domains. EHD3 localizes to early endosomes and may connect with Rab11 through Rab11-FIP2, and subsequently through its homo-oligomerization or hetero-oligomerization with EHD1 at the ERC to regulate the transport from early endosomes to the ERC [42].

The EHD1-binding partner MICAL-L1 is also a Rab8a effector [81,82]. Unlike most Rab effectors, association of MICAL-L1 with tubular membranes is independent of Rab8a. MICAL-L1 may recruit or stabilize EHD1 to tubular membranes and provide a link between EHDs and Rab proteins to coordinately regulate recycling to the plasma membrane [51]. The identification of additional proteins involved in the recycling pathways will be required to elucidate the exact sequence of events that occur in the course of recycling.

In addition to these three proteins, we have identified Rabankyrin-5/ANKFY1, a Rab5 effector required for macropinocytosis [83], as a novel EHD1-binding partner that contains the amino acid sequence NPFED [84]. Rabankyrin-5 partially co-localized with EHD1 on punctate membrane structures to regulate cargo internalization and recycling through both clathrin-dependent and -independent pathways [84].

It remains an open question, however, as to whether there is really significant cross-talk between Rabs and EHDs. One possibility is that the EHDs, whose absence from yeast indicates an evolutionary appearance later than the Rabs, evolved to take advantage of pre-existing effectors used by Rab family members. In this manner, EHD1 would have evolved to interact specifically with the Rab effectors Rabenosyn-5, MICAL-L1 and Rab11-FIP2. Additional studies will be necessary to elucidate whether these two endocytic regulatory protein families coordinate trafficking events or merely function in parallel.

SUMMARY

Over the past decade, EHDs have become known as integral regulators of endocytic trafficking, along with the Rab family of GTP-binding proteins. EHD and Rab protein families share some common features, such as nucleotide binding, transient association with membranes and interactions with motor proteins. However, they display distinct functions in regulating vesicle transport. The well-established model of Rab function is in membrane

tethering and fusion (Figure 3B). Rab proteins recruit tethering factors, which in turn interact with SNARE proteins and activate the formation of SNARE complexes, leading to membrane fusion [19,29].

In comparison, one of the models proposed for EHD function is the regulation of membrane fission (Figure 3A). According to this model, EHD-ATP dimers insert themselves into the hydrophobic lipid bilayer and induce membrane bending, while ATP hydrolysis induces a conformational change, which destabilizes the membrane and leads to membrane fission [33]. It is interesting that despite differences in function, Rab proteins and EHDs interact with each other through common effectors. These proteins interact with Rabs and EHDs through distinct domains and link Rab- and EHD-mediated pathways controlling endocytic trafficking.

Although significant progress has been made in understanding how Rabs and EHDs function in membrane trafficking, there are still many unanswered questions for both protein families. The diversity of Rabs reflects the complexity and multitude of endocytic traffic pathways in mammalian cells. Reconstitution of the Rab machinery *in vitro* may clarify our understanding of this complex process [85]. On the other hand, with the increasing number of Rab regulators and effectors being identified, it is important not only to characterize their individual roles but also to examine how they integrate cross-talk between multiple pathways. Less is known about how EHDs function in vesicle transport. A model has been proposed for EHD function in membrane fission [33], yet the mechanism remains to be proved. Another area that is still poorly understood is how EHDs interact with motor proteins. Identification of new EHD-binding partners may provide the necessary link by which EHD is connected with motor proteins to regulate microtubule-mediated vesicle transport [72].

In summary, Rabs and EHDs are key regulators of intracellular membrane trafficking. Further investigation of their distinct but complementary functions in regulating endocytic transport will improve our understanding of underlying mechanisms of endocytic transport.

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Abbreviations

CRMP2	collapsin response mediator protein-2
EE	early endosome
EH	Eps15 homology
EHD	Eps15 homology-domain-containing protein
ERC	endocytic recycling compartment

GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
GDF	GDI displacement factor
GEF	guanine-nucleotide-exchange factor
GGT	geranylgeranyltransferase
MICAL-L1	molecule interacting with CAsL-Like 1
NPF	asparagine-proline-phenylalanine
Rab11-FIP2	Rab11-family interacting protein 2
RILP	Rab7-interacting lysosomal protein
SNARE	soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor
TfR	transferrin receptor

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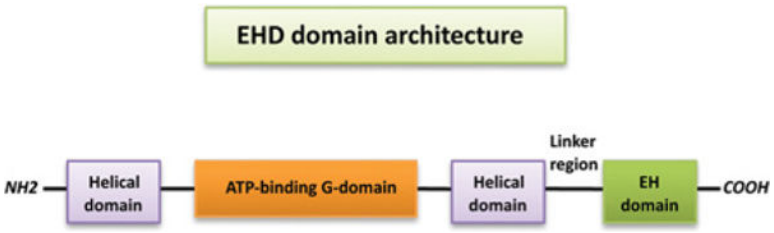

Figure 1. Schematic diagram of EHD domain architecture

EHDs contain two helical regions with an ATP-binding G domain in between. The C-terminal EH domain is connected with the helical domain by a 40-residue linker region.

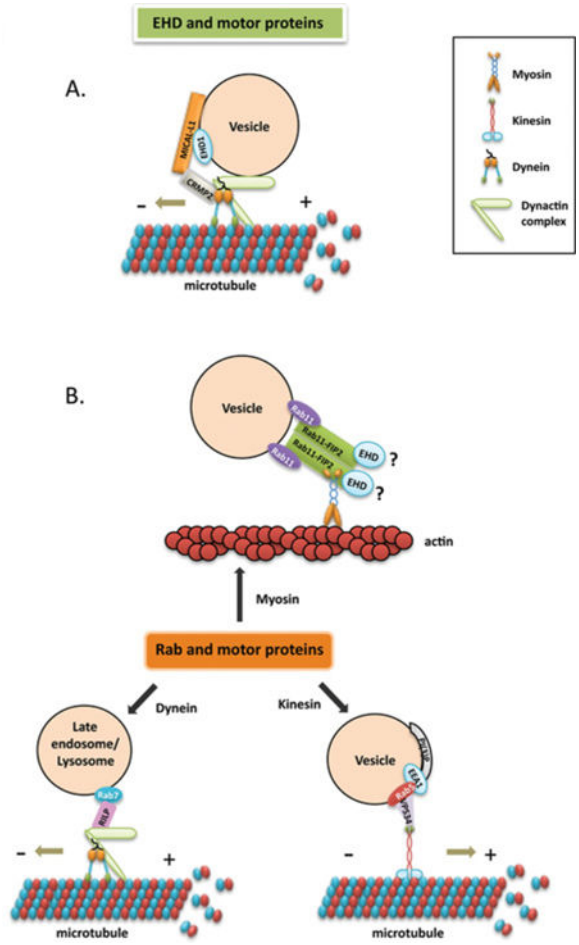

Figure 2. Interaction with motor proteins

(A) EHD and dynein. CRMP2 serves as a link between MICAL-L1-EHD1-associated vesicles and cytoplasmic dynein, and mediates microtubule-dependent vesicle transport. (B) Interactions between Rab proteins and either myosin, kinesin or dynein motors through their respective effectors. Rab11 interacts with myosin Vb through Rab11-FIP2, which is also an EHD-binding partner. Rab5 and its effector hVPS34 (human VPS34) regulate KIF16B (kinesin 16B) transportation of PI(3)P [PtdIns(3)*P*]-positive early endosomes. RILP binds to Rab7 and mediates vesicle transport by recruiting the dynein–dynactin complex.

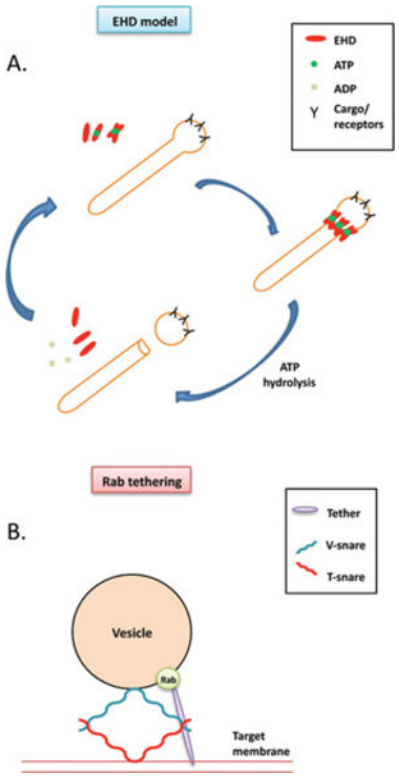

Figure 3. Models of EHDs and Rab proteins

(A) EHD ‘pinchase’ model. EHD-ATP dimers oligomerize along the tubular membrane. ATP hydrolysis induces a conformational change that destabilizes the membrane, which in turn leads to vesicle scission.

(B) Rab tethering model. Rabs recruit tethering factors, which in turn interact with SNARE proteins and activate the formation of SNARE complexes, leading to membrane fusion. Scale and stoichiometry are simplified for the illustration.