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Review

Rab3D: a regulator of exocytosis in non-neuronal cells

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Summary. Rab3D, a small Ras-like GTPase, is a key regulator of intracellular vesicle transport during exocytosis. It has been shown that Rab3 GTPases are abundant in cells with regulated secretory pathways and are thought to confer the specificity of docking and fusion during regulated exocytosis. Unlike other Rab3 isoforms, Rab3D is enriched in a number of non-neuronal tissues and is localised to secretory granules in the cytoplasm of these cells. The structure of Rab3D exhibits all of the conserved domains from the Rab family and also contains hypervariable N- and C-terminal regions. Rab3D undergoes post-translational isoprenylation and cycles between GDP- and GTP-bound forms. Apart from the factors involved in the Rab activation cycle, few Rab3D effector proteins have been identified to date. Nevertheless, it has long been suggested that Rab3D plays a role in regulated exocytotic processes as well as apically directed transcytosis. This review summarises the recent work on the biological function, structural integrity and molecular interactions of Rab3D in non-neuronal cells.

Key words: Rab3D, Osteoclast, Exocytosis, Vesicle transport, Isoprenylation

Introduction

Eukaryotic cells are highly compartmentalised and possess an elaborate network of vesicular traffic that is involved in a range of essential cellular functions. Over the last decade there has been an increase in the interest of the molecules that govern these processes. Small Ras-related Rab GTPases (Rabs) have emerged as important regulators of membrane trafficking. Rab proteins constitute the largest branch of the Ras superfamily with over 50 mammalian and 11 yeast proteins identified thus far (Pereira-Leal and Seabra, 2000). While many Rab proteins are ubiquitously expressed in mammalian cells, members of the highly homologous Rab3 subfamily (Rab3A, -B, -C and -D) are restricted to cells with regulated secretory functions (Touchot et al., 1987; Matsui et al., 1988; Baldini et al., 1992; Olkkonen and Stenmark, 1997).

Of the Rab3 isoforms there has been increasing interest in the role of Rab3D, originally isolated from mouse adipocytes (Baldini et al., 1992). Rab3D has been shown to play a key role during regulated exocytosis of non-neuronal systems (Olkkonen and Stenmark, 1997), including insulin, hormone and histamine secretion as well as secretion of bone resorbing enzymes by osteoclasts (Ohnishi et al., 1997; Roa et al., 1997; Iezzi et al., 1999; Zheng et al., 1999). In this review we focus on the current literature on Rab3D and its role in vesicle transport in a variety of cells.

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Structure

Rab3D is highly conserved among mammalian species (Baldini et al., 1992; Oberhauser et al., 1994; Nishio et al., 1999) and is located on chromosome 13, region A2-3 in humans. It consists of five exons spanning 10.6kb but the structural gene is contained in exons 2 through 5 with one canonical GTP-binding motif in each exon (Adachi et al., 2000). While its sequence remains conserved amongst its Rab3 counterparts, Rab3D is discernibly different from other Rab proteins (Baldini et al., 1992). In addition, Rab3D is largely differentiated from other Rab3 isoforms within the N- and C-terminal regions (Baldini et al., 1992). The variable C-terminal region of Rab3s is thought to confer the correct targeting of the proteins to specific locations (Chavrier et al., 1991), whereas the diversity of the N-terminal region is thought to be involved in interactions with unidentified elements within the secretory machinery (Steele-Mortimer et al., 1994; Iezzi et al., 1999).

Structurally, Rab3D, like all Rab3 GTPases, contains a number of conserved functional domains necessary for interactions with regulatory and/or effector proteins (Fig. 1). These domains include the effector region, the conserved switch I and II domains, the Rab complementarity-determining region (RabCDR) and the Cys-Ser-Cys (CSC) prenylation motif (Olkkonen and Stenmark, 1997; Ostermeier and Brunger, 1999).

The effector region is distinct between the Ras-related subfamilies and is analogous to a corresponding stretch in the Ras protein. It is thought to function in downstream signalling and interactions with accessory molecules. In addition, the effector region has been implicated in the determination of the different GTPases functional specificity (Brennwald and Novick, 1993; Dunn et al., 1993).

The RabCDR is a region of variable sequence found within a structurally conserved framework. Combined with the conserved switch mechanism the variable RabCDR enables Rab proteins to undergo specific interactions with a wide range of downstream effectors. Highly specific Rab protein/effector interactions are essential as multiple Rab systems are operating in the same cell (Dumas et al., 1999; Ostermeier and Brunger,

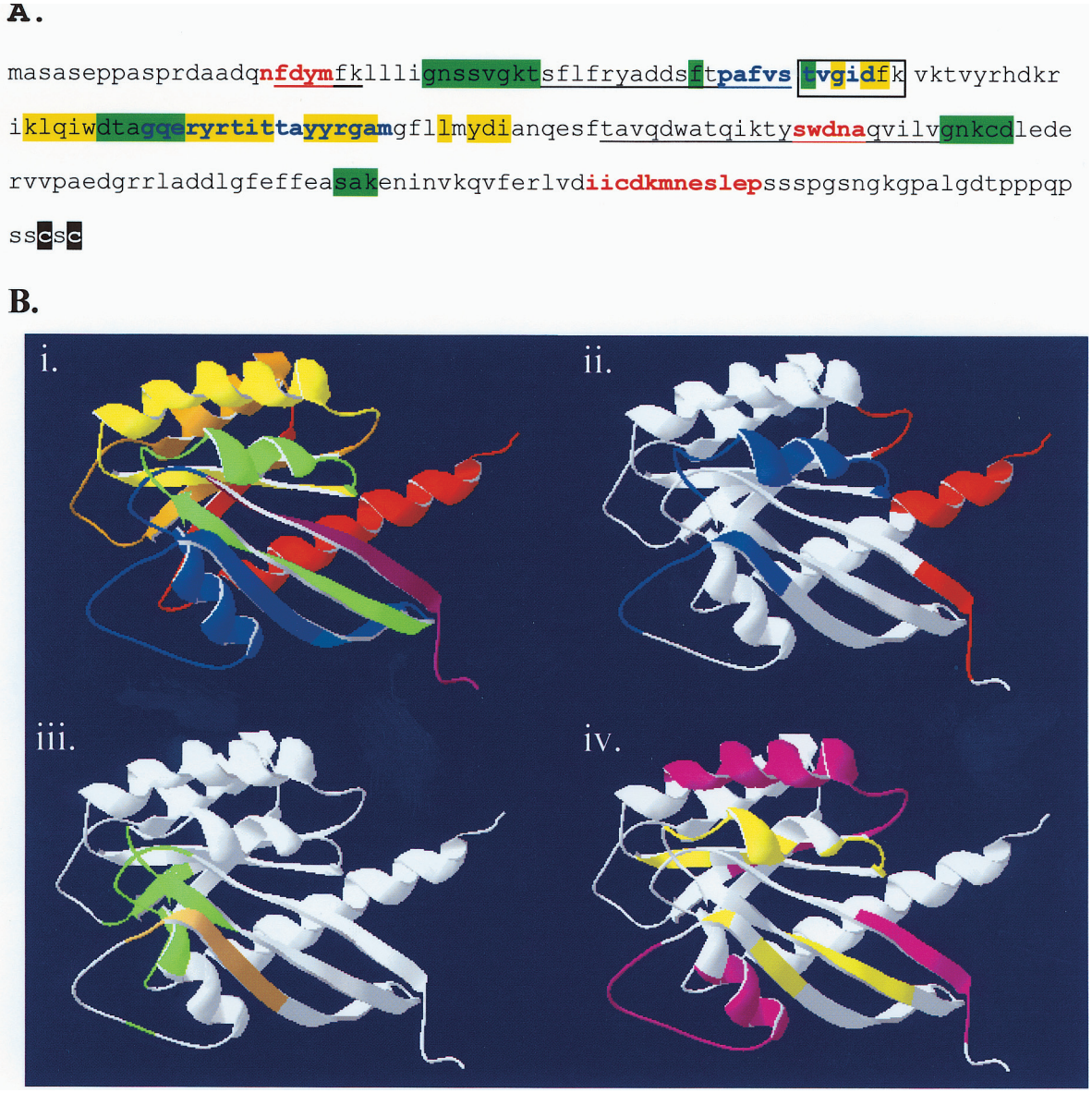

Fig 1. A. Rab3D amino acid sequence (GenBank Accession Number: AAA40026). The effector region is identified by a black box. The red amino acids indicate the three RabCDRs and the blue amino acids are the switch regions. The highly conserved nucleotide binding and hydrolysis motifs are highlighted in green and the Rab family sequence motifs are highlighted in yellow. The two cysteines involved in isoprenylation are highlighted in black. The Rab subfamily motifs are underlined. **B.** The putative crystalline structure of Rab3D. i. The six ß-strands and five α-helices highlighted in red, orange, yellow, green, blue and purple from the C-terminus to the N-terminus. ii. The switch I and II regions are highlighted in blue, while the RabCDR regions are red. iii. The effector domain is indicated in orange and the G-motifs are in green. iv. The Rab family motifs are yellow and the Rab subfamily motifs are pink.

1999).

Like other Ras related proteins, Rab proteins must undergo post-translation hydrophobic isoprenylation for membrane binding. The CSC motif of Rab3D is essential for this modification which is required for geranylgeranylation where two geranylgeranyl groups are added to the cysteines (Cremers et al., 1994).

Rab3D possesses a number of motifs that determine its function and its relation to other Rabs (Fig. 1A). Rab3D contains the five recently determined Rab family motifs (RabF) and the four Rab subfamiliy motifs (RabSF), of which three are highly conserved amongst the Rab3 isoforms (Pereira-Leal and Seabra, 2000). In addition, Rab3D contains the canonical g-motifs that are involved in GTP binding and hydrolysis (Fig. 1A), which are evenly distributed through exons 2-5 (Adachi et al., 2000).

Based primarily on the crystal structures of Rab3A and Ras (Pai et al., 1989, 1990; Milburn et al., 1990; Ostermeier and Brunger, 1999), we have generated a putative tertiary structure of Rab3D using SWISS-MODEL, an automated protein modelling server (http://www.expasy.ch/swissmod/SWISS-MODEL.html) (Peitsch, 1995; Guex and Peitsch, 1997; Guex et al., 1999). As shown in Figure 1B, the tertiary structure of Rab3D demonstrates the highly conserved fold of the GTP-binding domain (Olkkonen and Stenmark, 1997; Ostermeier and Brunger, 1999). The fold consists of a group of five loops that connect the ß-strands and the α-helices, which are the location of the nucleotide binding elements (Ostermeier and Brunger, 1999). Marked nucleotide-dependent conformational variability has been demonstrated in all of the regulatory GTPases that have been crystallised in both their GTP and GDP conformations in two regions. These two regions, switch I and II, are thought to undergo nucleotide-dependent conformational changes. There is also marked flexibility shown in the helix 3/loop 7 region (Olkkonen and Stenmark, 1997). It has been suggested that these local conformational changes between the GTP and GDP bound forms of the proteins control the activity of the GTPases (Dumas et al., 1999). The conformational differences lead to changes in the functional interactions with the other cellular elements.

Regulation

Cycling

Rab3D undergoes both a translocation and an activation/inactivation cycle (Simons and Zerial, 1993; Novick and Zerial, 1997; Takai et al., 2001) which require a number of accessory factors including GTPase exchange factor proteins (GEPs), GTPase dissociation inhibitors (GDIs) and GTPase activating proteins (GAPs) (Fig. 2). Rab GDIs maintain Rabs in the GDP-bound conformation in the cytosol. The release of the GDI from the GDP-Rab/GDI complex is accompanied by membrane targeting. Subsequently, GEP converts the GDP-bound Rab to its GTP-bound form by promoting the release of GDP and binding of GTP (Burton et al., 1993; Wada et al., 1997). At the final stage of the cycle, GAP converts the GTP-bound Rab to the GDP-bound conformation, resulting in the fusion of vesicles to the

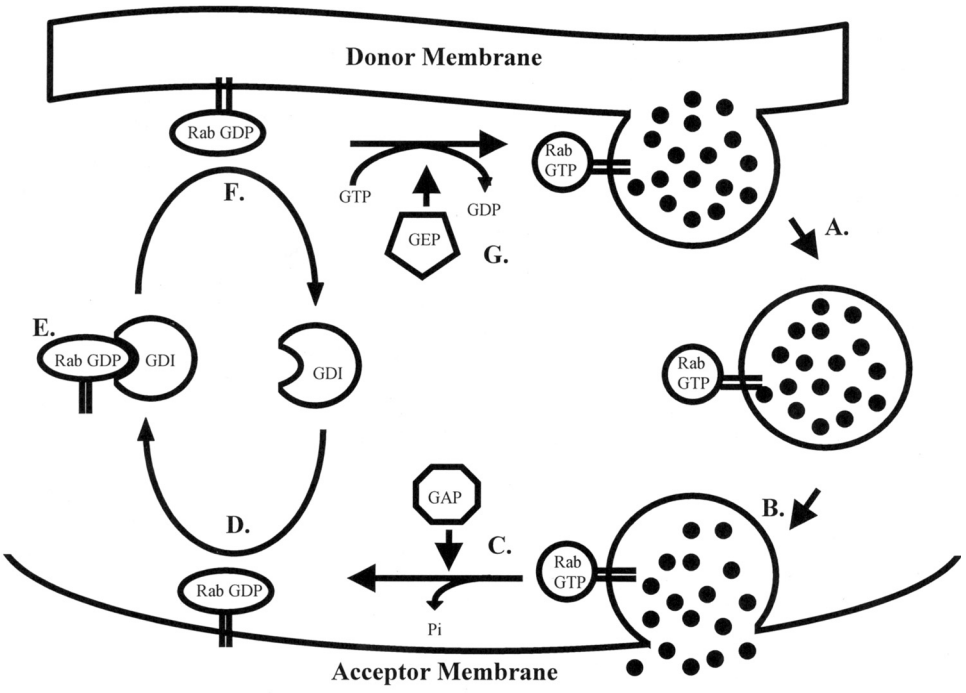

Fig. 2. The translocation and activation/inactivation cycle of the Rab GTPases. A circle represents Rab-GDP while Rab-GTP is depicted as an oval. The two lines connected to the Rab GTPase indicate the geranylgeranyl groups attached to the Rab proteins during isoprenylation. **A.** The vesicle, bound to Rab-GTP, buds from the donor membrane and travels through the cytoplasm to the acceptor membrane. **B.** This vesicle then binds to the membrane and it is thought that this step is mediated by the Rab-GTP. **C.** A GAP catalyses the GTP hydrolysis, deactivating the Rab protein and the release of the vesicles contents occurs. **D.** Rab-GDP is then released from the acceptor membrane with the assistance of a GDI. **E.** This Rab-GDP/GDI complex is how Rab proteins are maintained in the cytosol and recycled back to the donor membrane. **F.** Rab-GDP binds to the donor membrane displacing the GDI protein. **G.** A GEF activates the Rab protein by mediating the GDP/GTP exchange and the cycle begins again. (Modified from Novick and Zerial, 1997)

donor membrane (Novick and Zerial, 1997). Consequently, the GDP-bound Rab is returned to the cytosol as a soluble GDP-bound Rab-GDI complex.

Several recent studies have found that Rab protein subfamilies interact with specific members of these regulatory protein families (Fukui et al., 1997; Wada et al., 1997; Erdman and Maltese, 2001). A GEP that is specific for the Rab3 subfamily has been isolated and shown to be selectively active for Rab3A, Rab3C, and Rab3D (Wada et al., 1997). In addition, it has been demonstrated that different Rab GTPases associate preferentially with particular Rab-GDIs, however, specific Rab3D-GDI interaction has not yet been investigated (Erdman and Maltese, 2001). Furthermore, a Rab3 GAP that is specific for the Rab3 subfamily members has been characterised (Fukui et al., 1997). In short, Rab3D has a number of Rab3 subfamily specific proteins that regulate its activation/inactivation cycle.

Downstream effectors

Over the past decade a number of Rab3 effector proteins have been identified. However, only two of these effector proteins are consider to be possible effectors of Rab3D (Shirataki et al., 1993; Chung et al., 1999). Rabphilin-3A, the first Rab3 effector protein to be identified (Shirataki et al., 1993), is a 78kDa protein that preferentially binds to the GTP-bound conformation of Rab3A. Rabphilin-3A exhibits comparable binding constants with the GTP-bound forms of Rab3A, -B, -C, and -D (Chung et al., 1999) and is expressed in a range of cells including neuroendocrine cells and mast cell lines (Wada et al., 1994; Carroll et al., 2001). Rabphilin-3A is thought to serve as a regulator of the Rab3 proteins as it displays GEF activity on Rab3A and inhibits Rab3A GAP activity (Fujita et al., 1994; Clabecq et al., 2000). In addition, Rabphilin-3A has been shown to regulate exocytosis independently of Rab3 (Chung et al, 1999; Coppola et al, 1999; Joberty et al, 1999) and is not vital for the targeting of these GTPases to the synapses (Schluter et al., 1999). Conversely, Rabphilin-3A appears to require Rab3 proteins for its stability and synaptic localisation (Li et al., 1994).

The Rim proteins, Rim1 and Rim2, are Rab3 effector proteins which have been localised to active zones of synaptic systems (Castillo et al., 1997; Wang et al., 1997, 2000). Rim proteins, once thought to be exclusively expressed in neuronal tissue, have recently been isolated in pancreatic ß-cells (Iezzi et al., 2000). Rim may act as a regulator of synaptic-vesicle fusion by the formation of a GTP-dependent complex between docked synaptic vesicles and the synaptic plasma membrane (Wang et al., 1997). Rim-Rab3A interaction is not required for Rab3A-induced inhibition of exocytosis (Coppola et al., 1999). In contrast, Rim1 is thought to act as a positive regulator of exocytosis (Wang et al., 1997). Finally, while the region of the Rab3 proteins that binds Rim is highly conserved in the subfamily, it remains to be established whether it is a true Rab3D effector.

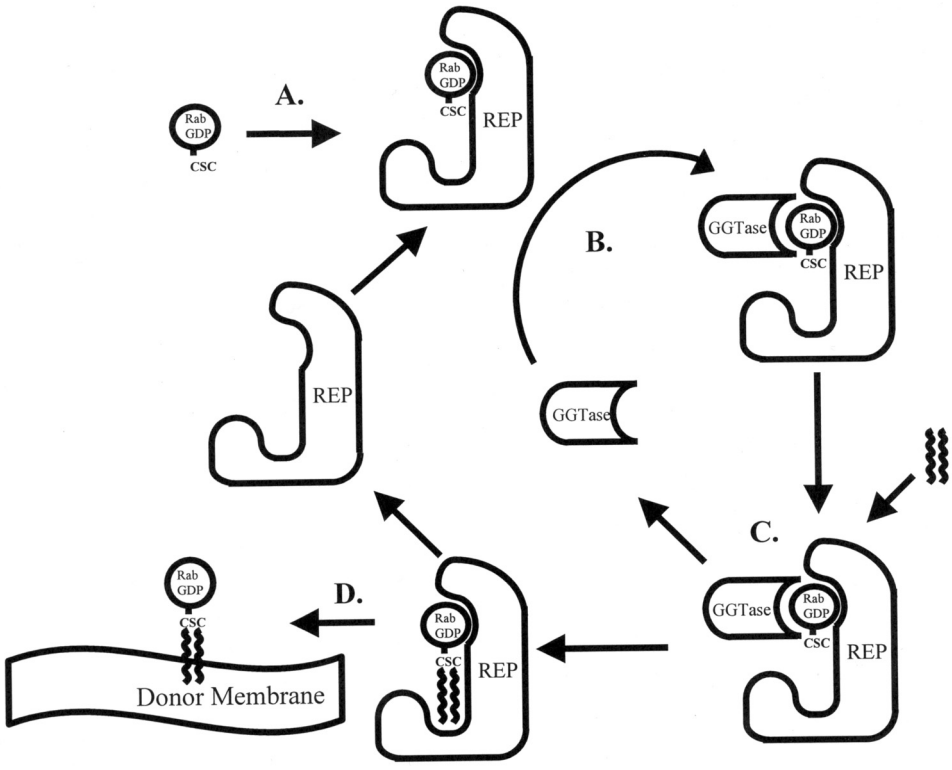

Fig. 3. Rab isoprenylation: **A.** The Rab escort protein, REP, binds the newly synthesised Rab-GDP. **B.** REP presents the GTPase to the Rab geranylgeranyl transferase, GGTase. **C.** GGTase attaches the two geranylgeranyl groups to the two C-terminal cysteine residues before dissassociating from the Rab GTPase/REP complex. **D.** REP remains associated with the GTPase after isoprenylation and transports it to the donor membrane. (Modified from Olkknen and Stenmark, 1997).

In short, although several downstream effectors of the Rab3 proteins have been identified, their role in vesicle transport remains unclear. Also, their interactions with the Rab3 proteins do not appear to be crucial and a more critical effector remains to be identified.

Isoprenylation

The post-translational isoprenylation of the C-terminal cysteines of Rab proteins is required for their membrane association and therefore their function (Casey and Seabra, 1996). It involves a 20-carbon geranylgeranyl moiety being attached to these cysteines in a consecutive manner by a thioether bond in a reaction catalysed by geranylgeranyl transferase II (GGTase) (Seabra et al., 1992). However, the C-terminal cysteines of the Rab proteins are not recognised by the Rab GGTase and an accessory protein known as the Rab escort protein (REP) is required to bind to the Rab proteins and present them to GGTase (Fig. 3) (Cremers et al., 1994). While Rab3D interacts with REP isoforms, REP-1 and REP-2, it is more efficiently prenylated in the presence of REP-1 (Cremers et al., 1994). REPs bind the unprenylated Rab proteins forming stable complexes (Alexandrov et al., 1994). It is thought that REPs act functionally as a GDI and mediate the movement of freshly geranylgeranylated Rab proteins to their appropriate membranes (Alexandrov et al., 1994; Wilson et al., 1996). In chief cells, cytosolic Rab3D coelutes with REP not GDI and in parotid acinar cells, cytosolic Rab3D interacts exclusively with REP even after agonist induced secretion (Raffaniello and Raufman, 1999; Chan et al., 2000). This is contrary to the popular belief that Rab proteins are only associated with REPs during isoprenylation.

Localisation and function

One of the characteristic properties of Rab GTPases is their localisation in distinct membrane compartments along the transport pathways of the eukaryotic cells. The Rab3 subfamily, including Rab3D, are predominantly localised to secretory vesicles that fuse with the plasma membrane by a calcium-triggered mechanism (Burgess and Kelly, 1987; De Camilli and Jahn, 1990; Sudhof et al., 1993; Olkkonen and Stenmark, 1997). Rab3D is enriched in a number of non-neuronal tissues and, in contrast with the other Rab3 isoforms, is only expressed in low levels in the brain (Baldini et al., 1992). Rab3D is localised to secretory granules of various exocrine secretory cells, including osteoclasts (Zheng et al., 1999) (see Table 1) and translocates from these vesicles to the plasma membrane during regulated exocytosis (Raffaniello et al., 1996; Tang et al., 1996; Tuvim et al., 1999; Chan et al., 2000; Martelli et al., 2000; Valentijn et al., 2000).

To date, the exact biological function of Rab3D in exocytosis still remains somewhat controversial, showing both positive and negative regulatory effects. Overexpression of Rab3D in pancreatic cells enhances cholecystokinin octapeptide-stimulated amylase secretion from intact acini and Ca^{2+}- and GTP-γ-S-triggered amylase secretion from streptolysin-O-permeabilized acini (Ohnishi et al., 1997). In contrast, Rab3D overexpression has been shown to inhibit degranulation in mast cells (Roa et al., 1997). In addition, Rab3D has been shown to inhibit the secretion of growth hormone (Chung et al., 1999), whereas others indicated that it enhances secretion (Martelli et al., 2000) in PC12 and chromaffin cells.

In the pancreas, Rab3D is localised to the zymogen granules of acinar cells. The actin coating of zymogen granules, in pancreatic acini, are part of a regulated sequence of events leading to exocytosis in the pancreatic acini. During the regulated secretion process the granules destined for secretion release Rab3D soon after they become actin coated as the majority of actin-coated granules lack Rab3D immunoreactivity (Valentijn et al., 2000). This release of Rab3D occurs prior to fusion of the secretory vesicles to the plasma membrane

Table 1. Rab3D cellular and subcellular localisation.

CELL TYPES	SUBCELLULAR LOCALISATION	REFERENCES
Adipocytes	Intracellular Membranes	Baldini et al., 1992, 1995
Mast Cells	Secretory Granules	Oberhauser et al, 1994; Tuvim et al., 1999
AtT-20 (Neuroendocrine)	Dense Core Granules	Martelli et al., 1995; Baldini et al., 1998
Gastric Chief Cells	Secretory Granules	Tang et al., 1996, Raffaniello et al., 1996
Pancreatic Acinar Cells	Zymogen Granules	Valentijn et al., 1996
Enterochromaffin-like Cells (Stomach)	Secretory Granules	Ohnishi et al., 1996
Acinar Cells (Lacrimal and Parotid Gland)	Secretory Granules	Ohnishi et al., 1996, Raffaniello et al., 1996
Paneth Cells (Intestine)	Secretory Granules	Ohnishi et al., 1996
Pancreatic ß-Cells	Secretory Granules	Iezzi et al., 1999
Granulocytes		Nishio et al., 1999
Chromaffin and PC12 Cells	Secretory Granules	Chung et al., 1999
Parathyroid Chief Cells		Huang et al., 1999
Osteoclasts		Zheng et al., 1999
Hepatocytes	Transcytotic Carriers	Larkin et al., 2000
Von Ebner's Glands	Secretory Granules	Field et al., 2001

as the GTPase is never detected at the apical membrane. Conversely, in the presence of actin-disrupting agents, Rab3D localises at the apical membrane indicating that Rab3D release is coupled to actin coating of granules (Valentijn et al., 2000). In short, these results indicate that Rab3D interacts with the actin cytoskeleton, directly or by way of an effector protein, and is involved in the step prior to docking and fusion in regulated exocytosis in the pancreatic acini.

Finally, it has been demonstrated that Rab3D is expressed in rat hepatocytes which are the classic models for constitutive exocytosis (Larkin et al., 2000). While Rab3D is mainly cytosolic in these cells there is some membrane-bound Rab3D found in the subcellular fraction that contains the transcytotic vesicles (Larkin et al., 2000). These Rab3D labelled compartments are enriched in transcytosed polymeric immunoglobulin A receptor (Larkin et al., 2000), suggesting that Rab3D may be involved in the apically directed transcytotic pathway in rat hepatocytes.

In conclusion, Rab3D is involved in the secretory pathways of eukaryotic cells. However, its precise function in these cells remains to be clarified. More research is still required to further clarify its biological function and its effector proteins. Rab3D knockout mice and Rab3D mutant studies may help further exploration of Rab3Ds role in regulating exocytosis.

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