Rab27a and melanosomes: a model to investigate the membrane targeting of Rabs

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Abstract
Rab proteins constitute the largest family within the Ras superfamily of small GTPases (>60 in mammals) and are essential regulators of transport between intracellular organelles. Key to this activity is their targeting to specific compartments within the cell. However, although great strides have been made over the last 25 years in assigning functions to individual Rabs and identifying their downstream effectors, the mechanism(s) regulating their targeting to specific subcellular membranes remains less well understood. In the present paper, we review the evidence supporting the proposed mechanisms of Rab targeting and highlight insights into this process provided by studies of Rab27a.

The Rab cycle couples membrane association/dissociation and activation status

Current long-standing models suggest that Rabs are synthesized in the cytosol where they bind with high affinity ($K_d$ in the picomolar range) to REP (Rab escort protein) [1–4]. REP may then present them to RGGT (Rab geranylgeranyltransferase), which catalyses the covalent attachment of one or usually two geranylgeranyl lipid(s) to C-terminus cysteine residue(s). REP may then target geranylgeranylated Rab to the cytoplasmic face of specific membranes. An alternative pathway has also been suggested where Rab associates with pre-assembled REP–RGGT complex, for prenylation and is then delivered to the membrane by REP [5,6].

At the membrane Rab is activated by exchange of bound nucleotide (GDP for GTP) [7]. Active Rab-GTP then recruits effector proteins from the cytosol to the membrane. These are a diverse group of proteins that include lipid kinases and phosphatases, molecular motors and tethering factors [1,2]. Thus Rabs may regulate vesicle budding, transport along cytoskeleton tracks and membrane fusion. After functioning Rab hydrolyses bound GTP to GDP and $P_i$, and effectors dissociate due to changes in the conformation of Switch I and II regions. Studied Rabs have relatively high affinities for nucleotides ($K_d$ in the nanomolar range) and relatively low intrinsic GTP hydrolysis and GDP dissociation rates ($\sim 0.1 \text{ min}^{-1}$), suggesting that extrinsic GAPs (GTPase-activating proteins) and GEFs (guanine-nucleotide-exchange factors) regulate their activity. Indeed, several RabGEF and RabGAP protein families have been identified that accelerate the Rab-GTP/GDP cycle in cells [8]. Rab-GDP may then be extracted from cellular membranes by the REP-related protein Rab–GDI (guanine-nucleotide dissociation inhibitor), thereby regulating Rab activity. GDI may also deliver Rab to membranes in vitro and these properties have fostered the idea GDI might allow recycling of Rab between acceptor and donor compartments thereby allowing Rabs to participate in multiple rounds of vesicular transport [7,9,10]. Thus initial REP-mediated delivery of nascent Rabs along with GDI-mediated extraction/delivery cycles may ensure the strict temporal and spatial regulation of Rab function that is vital for maintaining intracellular transport and organization. However, despite the obvious importance of these processes, the mechanism(s) regulating the specificity of membrane targeting of Rabs is still debated.

Rab targeting determinants

The CHD (C-terminal hypervariable domain)
Sequence comparison reveals that Rabs are most divergent over the C-terminal 35–40 residues, termed the CHD, suggesting that this region contains the information required to target Rabs to specific intracellular membrane [11]. Consistent with this early studies found that exchange of the Rab5 C-terminal 35 residues with the corresponding residues of Rab7 resulted in targeting of the Rab5CHD hybrid to Rab7-positive intracellular compartments in BHK (baby-hamster kidney) cells [11]. Similarly, a Rab6CHD chimera was re-targeted from the Golgi to the early endosomes and plasma membrane in BHK cells, although this was also dependent on the exchange of Switch II region ($\alpha_2/L5$) and $\alpha_3/L7$ (SF3) from Rab5 into Rab6 [12]. Equivalent studies of yeast secretory pathway Rabs [Ypt1, ER (endoplasmic reticulum) to Golgi transport and Sec4 post-Golgi transport]
revealed that the CHD of Ypt1 was necessary for it to target the Golgi and also sufficient to re-target Sec4 from post-Golgi membranes to the Golgi itself [13]. Consistent with this, later crystallographic analysis of the structure of geranylgeranylated Rab in complex with REP and Rab–GDI indicated that the CHD might be accessible to targeting factors [14,15].

Specific RabF (Rab family) and RabSF (Rab subfamily) motifs

However, in contrast with this, more recent studies indicate that CHD swapping does not affect subcellular localization of Rab1a, Rab2a, Rab5a, Rab7 and Rab27a, arguing against its role in targeting [16,17]. In contrast, these studies revealed that exchange of the specific RabF (conserved in all Rabs and involved in interaction with common regulators e.g. REP and GDI) and RabSF (conserved in closely related Rabs, e.g. Rab3 and Rab27, and involved in effector interactions) motifs between Rabs resulted in mistargeting of chimaeric Rabs, indicating their importance in targeting [14,18]. For instance, replacement of the SF3 or F4 motifs of constitutively active Rab5Q79L with those of Rab27a disrupted endosomal targeting of the chimaeric protein in HeLa cells. Meanwhile reciprocal replacement of the SF2 or SF3 motifs of Rab27a with those of Rab5a disrupted the secretory granule localization of Rab27a in pituitary-derived AtT20 cells. Interestingly, these studies revealed that chimaeric Rabs were isoprenylated and localized to perinuclear membranes that co-localize with the ER and Golgi, suggesting that this might be the primary site of membrane association of some Rabs from where they could be distributed to other specific compartments [16,17]. These observations also highlighted the differential requirement of specific RabSF and RabF sequences in the targeting of specific Rabs and suggest that targeting mechanisms may differ between Rabs.

Effector interactions

As RabSF and RabF motifs may contribute to Rab–effector interactions and also influence targeting (see above), it is possible that these interactions might be important in targeting or stabilization of membrane-associated Rabs. Consistent with this depletion of the Rab9 effector TIP47 destabilized Rab9 association with late endosomes in HeLa cells [17]. Meanwhile Rab9CHD and Rab9GCHD chimaeras in which the TIP47 interacting Rab9CHD was replaced with the corresponding region of Rab1 or Rab5 were mislocalized, confirming the importance of the Rab9–TIP47 interaction in targeting. Conversely, reciprocal Rab1CHD and Rab5CHD chimaeras were re-targeted to late endosomal membranes on co-expression of TIP47 [17]. These data support the possibility that interaction with downstream effectors stabilizes some Rabs on the membrane.

GDFs (GDI displacement factors)

Early in vitro studies of the delivery of Rab9 and Rab5 to purified membranes or membranes of permeabilized cells respectively revealed that these Rabs were delivered GDP-bound several minutes before being GTP-loaded [9,10]. This suggested that Rab–GDI dissociation precedes GEF-driven GDP/GTP exchange and predicted the existence of organelle/Rab-specific membrane-associated GDF as critical regulators of the membrane association of Rabs [7]. In line with this idea, biochemical studies identified a protease-sensitive membrane-associated factor that dissociated endosomal Rabs (Rabs 5, 7 and 9) from GDI, but did not manifest detectable GEF activity [19]. Contemporaneously, several Yips [Ypt (yeast Rab)-interacting proteins] or PRAs (prenylated Rab acceptors) were identified and investigated as putative GDFs as they interact with prenylated Rabs and harbour putative transmembrane domains [20–22]. Consistent with this it was found that the membrane protein Yip3 also known as PRA1 that partially co-localized with late endosomal Rab9, catalytically displaced GDI from Rabs 5, 7 and 9 (but not Rabs 1a and 2) and promoted their association with Yip3/PRA1-containing liposomes [23]. Crucially, it was reported that Yip3/PRA1 did not enhance the intrinsic rates of nucleotide exchange of several Rabs including Rab9 [23]. This suggested that Yip3/PRA1 might be a GDF for endosomal Rabs and led to the proposal that differentially distributed Yip/PRAs might act as compartment-specific GDFs recruiting Rabs from the cytosol on to membranes where they reside. Although other studies provide some evidence that Yips and related proteins may be regulating the specificity of membrane association of Rabs, many questions are outstanding regarding their role as regulators of Rab targeting [21,24,25]. Foremost of these is how a relatively small group of proteins (approximately ten in mouse), which each interact with several different Rab isoforms, regulate the specificity of targeting of >60 Rabs? Also it is unclear how the function of Yips in Rab targeting is regulated given that the subcellular distribution of these membrane proteins itself is likely to be regulated by vesicular transport.

GEFs

In contrast with studies indicating that GDI displacement and GEF-driven Rab activation are distinct events, more recent work raises the possibility that GEFs may regulate Rab targeting by themselves acting as GDFs. In particular, studies of the mechanism by which intracellular pathogen Legionella pneumophila subverts host intracellular trafficking pathways to promote its survival and proliferation reveal that a bacterial protein, DrfA, which is secreted into the host cytoplasm and localizes to the LCV (Legionella-containing vacuole) membrane, is a Rab1-specific GEF that recruits Rab1 to the LCV membrane [26,27]. Subsequent studies indicate that DrfA is a GDF for Rab1, and that the GDF activity of DrfA is dependent on its GEF activity [28,29]. These observations support a model in which GEF-mediated GTP loading of Rab occurs close to the target membrane, this then inhibits Rab–GDI interaction because of the lower affinity of GDI for Rab–GTP compared with Rab–GDP, and favours Rab recruitment to local membranes. Some evidence also supports the idea that mammalian GEFs may
regulate Rab targeting. For instance, siRNA (small interfering RNA) depletion of GEFs Varp (Rab21-specific) or Rab3GEP (Rab3/27-specific) disrupted Rab21 and Rab27a localization in HeLa cells and melanocytes respectively [30,31]. However, most identified GEFs are probably intrinsically cytosolic or peripheral membrane proteins meaning that their activity would have to be spatially regulated to ensure the targeting specificity of their substrate Rab(s) [8].

One hypothetical mechanism by which GEF activity might be so regulated is through networks of interactions, known as GEF cascades, which link the activity of Rabs within trafficking pathways. According to this model, active upstream Rab (e.g. Ypt32p that regulates intra-Golgi transport in yeast) may recruit the GEF (e.g. Sec2p GEF for Sec4p) for downstream Rabs (e.g. Sec4p) to the membrane as an effector thereby regulating the membrane recruitment of the downstream Rab. Evidence of GEF cascade in mammalian cells comes from studies of Rab5 and Rab7 GEF class C VPS (vacuolar protein sorting)–HOPS (homotypic fusion and protein sorting) complex plus Rab11 and Rab8 GEF Rabini8 [32–36]. However, it remains unclear how this process is initiated and the mechanism of recruitment of the first Rab or GEF. Interestingly, recent studies of DENN (differentially expressed in normal and neoplastic cells) domain-containing GEFs shed some light on this issue by revealing that some of these proteins manifest distinct subcellular localizations. For example, Rab35-specific GEF DENND1A/connecdenn localizes endocytic membranes in a clathrin-dependent manner, indicating that receptor activation and clathrin recruitment might in turn regulate downstream Rab activation and recruitment [37,38]. Future studies directed to understand the molecular mechanisms regulating GEF activity and the spatiotemporal relationship between GDI dissociation and nucleotide exchange may thus yield interesting insights into the mechanism of Rab targeting.

**Rab27a and the melanosome: a model to understand Rab targeting to organelles**

**Rab27a, melanocytes and melanosomes**

Skin melanocytes make pigmented melanosomes and then distribute them via a network of dendrites to neighbouring keratinocytes (~40 in humans), thus providing photoprotection [39–41]. In culture, melanocytes retain these characteristics and are large (>200 μm from dendrite tip to dendrite tip), flat (peripheral cytoplasm ~500nm z-depth) cells that synthesize numerous melanosomes. In melanocytes derived from black (C57BL/6) mice, their large (~500 nm diameter) black (eumelanin) core represents an excellent intracellular target organelle, easily visualized using bright-field microscopy. Together, these characteristic make melanocytes a favourable system for cell biological examination of Rab targeting.

Rab27 GTPases are conserved from Caenorhabditis elegans to humans and are widely expressed in cell types specializing in regulated secretion. Rab27 decorates the cytoplasmic face of the membrane of melanosomes in melanocytes, secretory granules in haemopoietic cells, including T-lymphocytes, mast cells and neutrophils, and neuroendocrine cells such as pancreatic β-cells, and regulates transport and exocytosis of these organelle [42,43]. Thus Rab27 function is linked to fundamental physiological processes underlying common diseases such as diabetes, inflammation, thrombosis and albinism. Also recent studies link elevated Rab27 expression and Rab27-dependent secretion with cancer cell metastasis and proliferation [44–46].

Rab27 is thought to play diverse roles in different cell types through interacting with different downstream effectors (~12 are known) [42]. In melanocytes, active Rab27a recruits effector Mlph (melanophilin) (also known as Slac2-a/Exophilin3), which binds motor protein MyoVa (myosin Va) to anchor melanosomes within peripheral actin-rich dendrites. This is essential for normal melanin transfer to neighbouring keratinocytes and thus skin and hair pigmentation [40,41]. Consistent with this, melanocytes lacking Rab27a, Mlph or MyoVa protein are unable to tether melanosomes in the dendrites and manifest perinuclear clustering (Figures 1A and 1B). More recently, Rab3GEP [also known as DENN/MADD (mitogen-activated protein kinase-activating death domain)] was identified as a GEF for Rab27a in melanocytes. Consistent with this idea, Rab3GEP depletion caused a decrease in Rab27a-GTP and perinuclear melanosome clustering, comparable with the phenotype seen upon depletion of Rab27a [31]. Thus melanosome distribution provides a convenient cellular read-out of Rab27a activity. Meanwhile, Rab27a targeting may be monitored by comparing the distribution of Rab, observed using fluorescence microscopy, with that of melanin, visible by bright-field microscopy (Figures 1C and 1D).

**Rab27a targeting to melanosomes**

Using these simple microscopic assays, it is possible to dissect the mechanism of Rab27 targeting in a cellular context. Early studies of Rab27a targeting focused on the possible role of post-translational geranylgeranylation. Geranylgeranylation (or prenylation) confers hydrophobicity to Rabs, thereby allowing them to associate with intracellular membranes [47]. However, Rabs present a variety of prenylation motifs (XXCC, XCCXX, XXCCX, CCXXX, XXCCX and XCCCX) suggesting that the arrangement and number of geranylgeranyl moieties added to a given Rab might influence its targeting. Interestingly, although Rab27 mutants bearing mono-prenylatable XXCCX or XXCCXX motifs remain cytosolic as predicted, a XCCCCX mutant was targeted to non-melanosomal membranes [48] and A.N. Hume, unpublished work). Thus, although double prenylation is essential for targeting to melanosomes, it is not required for membrane association. Following from this, and as indicated above, studies of the targeting of chimaeric Rabs revealed Rab27a targeting to melanosomes and secretory granules to be dependent on the integrity of the SF2 and SF3 motifs, but not the CHD [16]. Consistent with this, whereas Rab27 subfamily proteins share relatively high sequence similarity in RabSF and RabF motifs and target to melanosomes when...
Figure 1 | The function and intracellular targeting of Rab27a in mouse melanocytes

(A and B) Phase-contrast micrographs showing the distribution of melanosomes within melanocytes derived from wild-type and Rab27a-null mice. This illustrates the function of Rab27a in tethering melanosomes in the peripheral dendrites of these cells. (C) High-magnification micrograph showing the co-distribution of Rab27a (green) and melanosomes (false-coloured red) in melanocytes derived from the beige mouse that manifests large melanosomes (∼2 μm diameter). (D) Intensity of red and green signals along the dotted white line shown in (C). This highlights the presence of Rab27a on the limiting membrane of melanosomes.

expressed in melanocytes, sequence comparison reveals them to contain very little similarity within the CHD [49,50].

More recent studies have exploited Rab27a RabSF/F chimaeras in order to dissect the role of effector interaction and GEF-dependent activation in Rab27a targeting to melanosomal membranes. To probe the role of effectors, a battery of Rab27a RabSF/F swapped chimaeras were screened, using a yeast two-hybrid interaction assay, to identify those that failed to interact with known effectors. Although most mutants retained interaction with all or a subset of effectors, a mutant in which both the RabSF1 and RabF4 of Rab27a were replaced with those of Rab5a (Rab27aSF1F4) was identified as being incompetent for interaction with known effectors [51]. Strikingly, this mutant, as found for wild-type Rab27a, was efficiently targeted to melanosomes. Conversely, it was found that the mistargeted Rab27aSF2 mutant was competent for effector interaction. Together these observations indicate that effector interaction is not an absolute requirement for targeting of Rab27a in melanocytes. This is consistent with the finding that Rab27a associates with melanosomes in melanocytes that lack the effector Mlph (melan-in) [52].

To test the role of GEF activity in targeting, immunofluorescence microscopy and subcellular fractionation were used to determine the intracellular localization of Rab27a in Rab3GEP-depleted melanocytes. Intriguingly, these studies revealed that, although targeting to melanosomes was disrupted, Rab27a remained associated with membranes in Rab3GEP-depleted cells [51]. These observations indicate that GDP/GTP exchange is essential for targeting of Rab27a to melanosomes and suggest that, in wild-type cells, Rab27a-GDP may be initially delivered to intermediate/non-melanosomal membrane before GTP loading triggers delivery to and stabilization at the melanosome. However, the results of in vitro assays revealed that Rab3GEP can catalyse nucleotide exchange on both targeted (Rab27aSF1F4) and mistargeted (Rab27aSF2) mutants with efficiency similar to that of wild-type Rab27a. Overall, these observations indicate that while Rab3GEP-driven Rab27a activation is essential, it is by itself insufficient to allow melanosomal targeting of Rab27a. This suggests that other essential factors regulate Rab27a targeting to melanosomes. Future studies should aim to identify such factors.
Closing remarks
In conclusion, 25 years after their discovery, it is remarkable that the molecular mechanisms governing the targeting of Rabs to their subcellular resides remain to be fully understood. Indeed the differing models proposed to date highlight that Rab targeting is a highly complex process that may require several factors and underline that much remains to be discovered in this fundamental area of cell biology.

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