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MINI-REVIEW

Post-translational modifications: How to modulate Rab7 functions

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ABSTRACT

The small GTPase Rab7 is the main regulator of membrane trafficking at late endosomes. This small GTPase regulates endosome-to-trans Golgi Network trafficking of sorting receptors, membrane fusion of late endosomes to lysosomes, and autophagosomes to lysosomes during autophagy. Rab7, like all Rab GTPases, binds downstream effectors coordinating several divergent pathways. How cells regulate these interactions and downstream functions is not well understood. Recent evidence suggests that Rab7 function can be modulated by the combination of several post-translational modifications that facilitate interactions with one effector while preventing binding to another one. In this review, we discuss recent data on how phosphorylation, palmitoylation and ubiquitination modulate the ability of this small GTPase to orchestrate membrane trafficking at the late endosomes.

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Introduction

The small GTPases of the Rab family play crucial roles in determining the identity and destination of vesicles. As such, they are key regulators of the formation, trafficking, and fusion of transport vesicles at the endoplasmic reticulum (ER), Golgi apparatus and early and late endosomes.[1] Cells tightly regulate the function of Rab GTPases through various mechanisms. Indeed increasing evidence suggests that alterations in the physiological functions of Rab GTPases can lead to the development of a wide range of diseases, spanning from cancer[2] to neuropathy[3] and neurodegenerative disorders.[4]

After their cytosolic synthesis, Rab GTPases bind Rab Escort Protein (REP)[5] and are presented to the geranylgeranyltransferase (RabGGTase)[6] to be prenylated (geranylgeranylated) on their C-terminal cysteines.[7] Prenylation is a non-reversible post-translational modification consisting in the attachment of a lipidic moiety to a cysteine residue. It was the first post-translational modification identified on Rab GTPases and is required for Rabs to bind membranes as mutations affecting C-terminal cysteine prenylation preclude membrane localization of the protein, which appears almost completely localized in the cytosol.[8] Rab GTPases cycle between a GTP-bound active state and a GDP-bound inactive state. In their inactive state, Rab GTPases are bound by a guanine-nucleotide dissociation inhibitor (GDI) that masks the prenylated tail and keeps Rabs soluble in the cytosol.[9]

The exact mechanisms governing membrane translocation of Rabs, as well as how specific membrane targeting is achieved is not completely understood. Evidence indicates that guanine exchange factors (GEFs) are able to induce GDI release and Rab targeting onto specific membranes,[10,11] suggesting that each GEF activates and recruits a specific Rab at a defined subcellular compartment.[12] This GDP to GTP switch also enables Rab GTPases to interact with specific downstream effectors.[13] GTPase activating proteins (GAPs) terminate the activity of Rab GTPases by hydrolyzing GTP to GDP.[14] In a GDP bound form, Rabs bind to GDI, are returned to the cytosol, and the cycle can begin anew.

The small GTPase Rab7 is a regulator of membrane trafficking at the late endosome and also plays other key roles by interacting with a variety of downstream effectors. Rab7 regulates the spatiotemporal recruitment of retromer, an evolutionary conserved multimeric complex composed of a trimer of vacuolar protein sorting (Vps)-26, Vps35, and Vps29, and of a dimer composed of a varying combination of sorting nexins (SNX).[15,16] In mammalian cells, retromer is responsible for the retrieval of Vps10-domain family receptors (Sortilin, SorLA, SorCS)[17,18] and cation-independent mannose phosphate receptor (CI-MPR) from the late endosome to the trans-Golgi Network (TGN).[19,20] In what appears to be a Rab7-independent pathway, retromer is also responsible for recycling of integral membrane proteins from the

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endosome to the plasma membrane.[21] Rab7 is also required for the degradation of cell surface receptors such as the Epidermal Growth Factor Receptor (EGFR).[22,23] Finally, Rab7 also participates in autophagosome-lysosome fusion through the homotypic fusion and vacuole protein sorting (HOPS) complex,[24] thereby regulating autophagy. For more detailed information on Rab7 functions, we refer the reader to a recent review by Guerra and Bucci.[25] How Rab7 is able to specifically interact with several effectors to regulate these various pathways is still not completely understood. In the last few years, the role of post-translational modifications including phosphorylation, ubiquitination and palmitoylation, in modulating Rab7 function has been increasingly explored. In this review, we will highlight recent advances on how post-translational modifications regulate Rab7 function.

Phosphorylation

Phosphoproteomic analysis identified at least two phosphorylatable sites on Rab7, serine 72 (S72) and tyrosine 183 (Y183).[26,27] Although the phosphorylation of these two residues has now been demonstrated, it is still not fully understood how this post-translational modification at these sites modulate Rab7 function(s) and which kinase(s) and phosphatase(s) are involved. However recent work has begun to unravel the role of phosphorylation on Rab7 function.

Serine 72

Phosphorylation on Serine 72 appears to negatively regulate Rab7 activity in the cell. Indeed, the phosphomimetic mutant Rab7^{S72E}, which mimics a constitutively phosphorylated protein at this site, does not localize to endosomes and is almost completely cytosolic.[28] On the contrary, the phosphonull Rab7 mutant Rab7^{S72A}, that cannot be phosphorylated, shows a similar localization to wild-type Rab7.[28] Functionally, HeLa cells transfected with Rab7^{S72E} demonstrate delayed EGFR degradation, compared to non-transfected cells or cells expressing wild-type Rab7.[28] In turn, this resulted in increased EGFR signaling.[28] Further supporting a role of phosphorylation at S72 functioning as a negative regulator of Rab7 function, Rab7^{S72E} does not interact with its effector Rab interacting lysosomal protein (RILP) as efficiently as wild-type Rab7 or Rab7^{S72A}. Taken together, this data suggests a possible role of S72 phosphorylation as a molecular switch that, together with GAP-mediated GTP to GDP hydrolysis, contributes to termination of Rab7 activity in the cell.

Tyrosine 183

A recent publication showed that the phosphorylation of Y183 prevents the protein from localizing to endosomes. Indeed, phosphomimetic Rab7^{Y183D} was found in the cytosol compared to wild-type Rab7 or phosphonull Rab7^{Y183F} which showed punctate staining.[28] However, a subsequent study found phosphomimetic Rab7^{Y183E} displaying a similar pattern of localization to phosphonull Rab7^{Y183F}, suggesting that constitutive phosphorylation of Y183 did not interfere with the ability of Rab7 to bind endosomal membranes.[29] The discrepancy in these results could possibly be explained by the use of two different phosphomimetic mutations, Y183D versus Y183E. However, in both cases, quantification experiments were not performed. A robust statistical analysis of the distribution of these mutants or membrane separation assays could be used to determine the localization of these mutants, in side-by-side comparison to determine whether of not phosphorylation at Y183 affects membrane binding.

Functionally, three studies have investigated the impact of Y183 phosphorylation on the degradation kinetics of EGFR. In two cases, it was found that overexpressing phosphomimetic Rab7^{Y183D} or Rab7^{Y183E} in HeLa cells impaired EGFR degradation.[28, 29] However, in contrast to these finding, another group reported that phosphorylation on Y183 is required to efficiently degrade EGFR.[30] Indeed, these authors demonstrated that subsequent to EGF stimulation, EGFR promotes Rab7^{Y183} phosphorylation enabling its degradation in lysosome and preventing its recycling to the plasma membrane.[30] In that same study, expression of the phosphonull mutant, Rab7^{Y183F}, blocked EGFR degradation and sustained extracellular signal–regulated kinases (ERK) signaling.[30] It is important to point out that all three groups performed experiments in cell expressing endogenous Rab7 that could affect the data obtained, especially in the overexpression studies.

Studies to determine the role of Y183 phosphorylation in mediating downstream effector interactions found that Y183 phosphorylation inhibits Rab7 binding with RILP. Indeed, the phosphomutant Rab7^{Y183F} was able to co-immunoprecipitate more RILP than wild-type Rab7, while the phosphomimetic mutants Rab7^{Y183D} and Rab7^{Y183E} do not co-immunoprecipitate this effector.[28,29] If Y183 mediates membrane localization as was shown for Rab7^{Y183D}, and membrane localization is a prerequisite for activation, this would explain why the interaction with RILP was lost and this would also suggest that all effector binding would be lost. If phosphorylation at Y183 does not affect membrane binding as was shown with Rab7^{Y183E}, this would suggest that phosphorylation at this site could be used to prevent the Rab7/RILP interaction and redirect the activity of Rab7 toward a different

effector(s), while not affecting its GTP loading. It will be important to further characterize the functional role of Y183 phosphorylation by testing more effector interactions and functional pathways where Rab7 plays a crucial function.

Kinases and Phosphatases

To date, little is known about the kinases and phosphatases regulating the Rab7 phosphorylation cycle. For example, no kinase(s) have been identified that would phosphorylate S72. However, Src kinase has been identified as responsible for the phosphorylation on Y183. Indeed, a significant decrease in the phosphorylation level of Rab7 was observed in cells treated with an inhibitor of the Src-kinase family or shRNA depletion of Src kinase.[29]

The phosphatase and tensin homologous 10 (PTEN) seems to have a role in de-phosphorylating Rab7 on both serine 72 and tyrosine 183[28] as shRNA-mediated knockdown of PTEN was associated with increased Rab7 phosphorylation on both S72 and Y183. Moreover, depletion of PTEN induced a re-localization of the small GTPase from late-endosomes to the cytosol, recapitulating the subcellular localization of the Rab7 phosphomimetic mutants.[28] More work will be required to identify and characterize other enzymes in the Rab7 phosphorylation cycle and identify which functions of Rab7 these proteins regulate.

Ubiquitination

Ubiquitination is the attachment of a small protein, ubiquitin, to a lysine residue of a target protein substrate.[31] This reversible post-translational modification is mediated by the coordinated action of three enzymes, E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-ligases, while a family of deubiquitinating (DUBs) enzymes is responsible for ubiquitin deconjugation.[32,33] Ubiquitination regulates several aspects of cellular function, such as endocytosis,[34] intracellular signaling pathways[35] and protein degradation in lysosomes.[36,37] A target protein can be mono or poly-ubiquitinated at one or multiple sites and linear or branched ubiquitin chains can be attached, finely modulating the substrate function.[38] Global analysis of the ubiquitinated proteome identified Rab7 lysine K38, K191 and K194 as possible target for ubiquitination.[39–41] Interestingly, Rab7 ubiquitination is mediated by Parkin, which belongs to the family of E3 ubiquitin ligase. Loss-of-function mutations on *parkin* have been linked to the development of familial, early onset Parkinson's disease (PD), demonstrating a possible role of Rab7 in this disease. Parkin mediates the ubiquitination of all three ubiquitinatable Rab7 lysine residues, with preference for K38. In particular this modification appears to be important to stabilize Rab7, favoring the interaction of the small GTPase with its effector RILP. Indeed, mutation of K38 on Rab7 or mutations inactivating the ubiquitin ligase-activity of Parkin are associated with reduced binding between Rab7 and RILP.[40] Furthermore, $Rab7^{K38R}$ mutant is displaced to the cytosol, suggesting that K38 ubiquitination is required for efficient translocation of Rab7 to membranes.[40] Functionally, the expression of $Rab7^{K38R}$ partially recapitulates the phenotype associated with Parkin loss of function, highlighting the importance of a strictly regulated control of Rab7 activity in the context of PD.[25,40] To date, no DUBs have been identified as Rab7 deubiquitinating enzymes.

Palmitoylation

Palmitoylation (also known as S-Acylation) is the reversible attachment of a palmitate group to a cysteine residue of a protein via a reversible thioester bond.[42] This post-translational modification can modulate the function of a protein in different ways including stability, protein-protein interactions and membrane binding.[43] Palmitoyltransferases (PATs) are enzymes that mediate palmitoylation and contain a conserved catalytic domain, characterized by the amino acid sequence aspartic acid-histidine-histidine-cysteine.[44] In humans, 24 PATs (also referred as DHHCs) have been identified,[44–46] and proteomic analysis of S-acylated proteins in mammals revealed that several hundred proteins are palmitoylated.[44] We do not yet know the substrate specificity of each DHHC, but it is clear that some proteins can be palmitoylated by different DHHCs, while others require the action of a defined enzyme.[44] Much less is known about the mechanisms regulating protein depalmitoylation. Acyl-Protein thioesterases (APT) 1 has been shown to have a role in G protein alpha subunit, SNAP-23 and H-Ras depalmitoylation,[47–50] while APT2 has been shown to be responsible for deacylation of GAP-43.[48] Recently, members of the ABHD family have been identified as thioesterases for N-Ras and PSD-95,[51,52] but little is known on the cellular localization and substrate specificity of these enzymes.

We have recently shown that Rab7 is palmitoylated on two cysteine residues, C83 and C84. This modification is not required for membrane binding as non-palmitoylatable Rab7, $Rab7^{C83,83S}$, was still membrane bound and localized to endosomes.[8] Palmitoylation regulates the ability of Rab7 to interact with and recruit retromer to endosomes.[8] Rab7 palmitoylation is essential for efficient retromer function in cells as the expression of $Rab7^{C83,84S}$

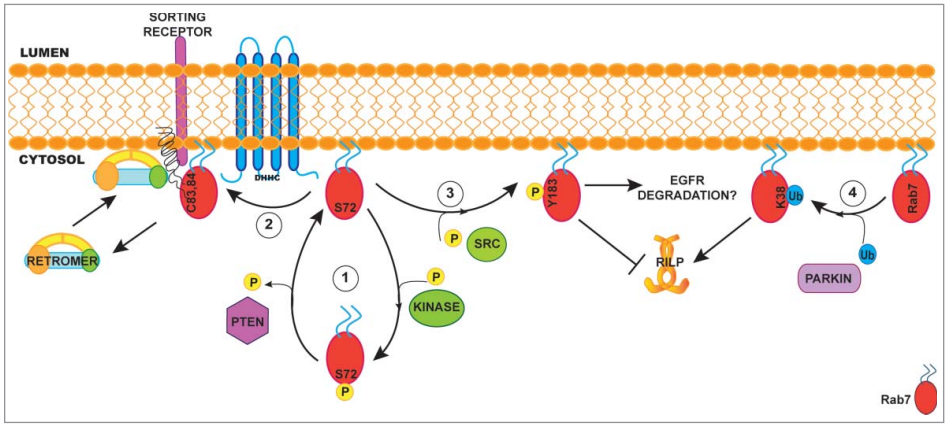

Figure 1. Role of post-translational modifications in modulating Rab7 functions. (1) Phosphorylation on Serine 72 by a non-identified kinase displaces Rab7 from the membrane to the cytosol. Here, the phosphatase PTEN can remove the phosphorylation on Serine 72 enabling Rab7 to bind the membrane again. (2) On the membrane, Rab7 can be palmitoylated by a yet unknown palmitoyltransferase. Palmitoylation on cysteines 83 and 84 is required to recruit retromer at the late endosome to mediate sorting receptor retrieval to the trans-Golgi Network. (3) Src mediated phosphorylation of Y183 blocks Rab7 interaction with its effector RILP. The role of this modification on EGFR degradation is controversial as some groups found that phosphorylation is required for degradation while other found it must not be phosphorylated. (4) Ubiquitination of K38 mediated by the E3-ligase parkin stabilizes Rab7 on the membrane and is required for the interaction with the Rab7 effector RILP. In this schematic view, Rab7 is represented with one post-translational modification at time, but we cannot exclude the possibility that palmitoylation, phosphorylation and ubiquitination are mutually exclusive. In fact, it is feasible that various combinations of post-translational modifications determine the specific function of Rab7 on late-endosomal membranes.

in a Rab7-KO HEK293 cell line was not able to properly recruit retromer to late endosomes. This lead to impaired endosome-to-TGN cargo receptors recycling that ultimately caused the miss-localization of lysosomal enzymes, which are secreted into the extracellular media.[8]

Interestingly, palmitoylation is required for the Rab7/retromer interaction and recruitment, but is not required for the Rab7/RILP interaction. Indeed, not only did the non-palmitoylatable mutant Rab7^{C83,84S} interact with RILP as efficiently as wild-type Rab7, but its expression in Rab7-KO cell restored the degradation of EGFR as efficiently as wild-type Rab7, suggesting that palmitoylation is involved specifically in regulating the Rab7/retromer interaction and function.[8]

There is no data yet on the enzymes regulating the Rab7 palmitoylation/depalmitoylation cycle. DHHC5 was found in a screen to identify proteins involved in endosome-to-TGN trafficking[53] and it would be interesting to investigate if Rab7 is one of the targets of this enzyme.

Conclusion and Perspectives

In the last few years, increasing evidence has pointed to how the functions of Rab7 are modulated by different post-translational modifications. Indeed the combination of palmitoylation, phosphorylation and ubiquitination enables the protein to interact specifically with one effector and not others, making Rab7 extremely versatile and able to coordinate several different pathways at the late endosome. We are only at the beginning of our understanding of the mechanisms behind Rab7 post-translational modifications. The enzymes involved in these processes have not been fully identified or characterized and the function of these modifications requires further work. Although one kinase (Src),[29] one phosphatase (PTEN)[28] and an E3 ubiquitin-ligase (Parkin)[40] have been identified (Fig. 1), the enzymes in the palmitoylation cycle have not and we predict that other kinases, phosphatases and ubiquitin ligases may yet be identified and characterized. Once we have a greater understanding of the players involved in controlling these modifications, this machinery could be used as pharmaceutical target to differentially modulate the function of Rab7.

Rab7 and its downstream effectors such as retromer have been implicated in neurological diseases such as Charcot-Marie-Tooth disease[54] and in the development of neurodegenerative diseases such as Alzheimer's (AD),[55] Parkinson's (PD)[56] and Batten disease (BD).[57] Indeed, even small reductions in retromer function can progressively impair the lysosome degradative system leading in the long term to neurodegeneration. Post-mortem analysis of brain sections of AD patients revealed a decrease in the expression level of retromer subunits.[58,59] Interestingly, Rab7 expression was upregulated in AD patients.[60] Moreover, mutations affecting subunits of the retromer heterotrimer have been linked to familial PD[61–63] and atypical Parkinsonism.[64] Due to

its crucial role in the pathogenesis of neurodegenerative disorders, modulating the function of retromer via Rab7 post-translational modification could offer a novel therapeutic approach to treat these diseases. *In vitro*, the use of pharmaceutical chaperones stabilizing retromer has been shown to improve retromer functions ameliorating AD[65] and PD phenotypes[66] highlighting the feasibility of using this approach.

Disclosure Statement

The authors declare no conflicts

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