Site-directed mutagenesis, in vivo electroporation and mass spectrometry in search for determinants of the subcellular targeting of Rab7b paralogue in the model eukaryote Paramecium octaurelia

E. Wyroba, P. Kwainski, K. Miller, K. Kobylecki, M. Osinski
Nencki Institute of Experimental Biology of Polish Academy of Sciences, Warsaw, Poland

Abstract

Protein products of paralogous genes resulting from whole genome duplication may acquire new functions. The role of post-translational modifications (PTMs) in proper targeting of Paramecium Rab7b paralogue (distinct from that of Rab7a directly involved in phagocytosis) was studied using point mutagenesis, proteomic analysis and double immunofluorescence after in vivo electroporation of the mutated protein. Here we show that substitution of Thr200 by Ala diminished the incorporation of [P32] by 37% and of [C14-]UDP-glucose by 24% into recombinant Rab7b_200 in comparison to the non-mutagenized control. Double confocal imaging revealed that Rab7b_200 was mistargeted upon electroporation into living cells in contrast to non-mutagenized recombinant Rab7b correctly incorporated in the cytosome area. Using nano LC-MS/MS to compare the peptide map of Rab7b with that after deglycosylation with a mixture of five enzymes of different specificity we identified a peptide ion at m/z=677.63+ representing a glycan group specific to Thr200. Based on its mass and quantitative assays with [P32] and [C14]UDP-glucose, the suggested composition of the adduct attached to Thr200 is (Hex)1(HexNAc)1(Phos)3 or (HexNAc)1(Deoxyhexose)1(Phos)1(Hex)1. These data indicate that PTM of Thr200 located in the highly conserved C-region of Paramecium octaurelia Rab7b is crucial for the proper localization/function of this protein. Moreover, the two Rab7 paralogues differ also in another PTM: substantially more phosphorylated amino acid residues are in Rab7b than in Rab7a.

Introduction

Divergence of genes arose by whole genome duplications (WGD) may endow their products with new functions.1 Since it is known that post-translational modifications (PTMs) may affect protein interactions,2 we tested whether PTMs could contribute to the neofunctionalization of the rab7b gene product in the model eukaryote Paramecium octaurelia. Based on extensive phylogenetic analyses comprising 210 proteins we reported earlier that Rab7 proteins evolved before the radiation of main supergroups of Eukaryota and are widely distributed in almost all of them.3 We cloned two Rab7 genes from Paramecium octaurelia,4 known to undergo at least three successive WGDs.5

Paramecium octaurelia Rab7a and Rab7b, displaying 62.3-63.3% identity with human Rab7,4 have distinct localizations, expression and functions.6 Silencing of Rab7a (Mr = 22.5 kDa) suppressed phagosome formation by 70% and impaired their acidification. Ultrastructural analysis with double immunogold labeling revealed that this effect was due to a lack of V-ATPase recruitment. During phagocytosis expression of Rab7a was almost 5-fold higher than that of Rab7b. Rab7b (Mr =25 kDa) associated with microtubule bundles and structures supporting the oral apparatus.4 No phenotypic effects of Rab7b depletion by RNAi have been noticed. In 2D gel electrophoresis two Rab7b immunoreactive spots of slightly different pI (~6.34 and ~6.18) were observed and a single spot of pI ~6.34 for Rab7a. Both ProQ Emerald staining and ConA overlay assay of immunoprecipitated Rab7b indicated its likely glycosylation in accordance with its faster electrophoretic mobility upon deglycosylation.4

The two Paramecium octaurelia Rab7 paralogues differ from each other by five amino acids out of 206: four in the C-terminal hypervariable region that participates in determinants of specificity of Rab7 interactions with membranes, its localization within the cell7 and is an important determinant of effector binding.8 The fifth diverged amino acid residue in position 140 (Ser in Rab7b and Ala in Rab7a)9 is located in the region of α-helix with the highest frequency of secondary structure elements.9

Based on in silico modeling (NetOGlyc 4.0 analysis tool)10 Thr200 is the unique site in Rab7b that may undergo O-glycosylation distinguishing this protein from Rab7a. Therefore this amino acid was changed to alanine to prevent PTM and site directed mutagenesis was confirmed by LC-MS/MS. We looked into the details of this machinery in recombinant system and created the mutagenized form of Rab7b not undergoing PTM to follow its intracellular targeting and properties.

Materials and Methods

Reagents

All reagents were of the highest purity available from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Plasmid construction and site-directed mutagenesis

Since cDNA from Paramecium octaurelia cannot be used to express a protein in E. coli due to the different genetic code of Ciliates, a Rab7b coding sequence optimized for protein expression in E. coli flanked by EcoRI and XhoI sites was synthesized commercially (Mr. Gene GmbH, Regensburg, Germany). This sequence was introduced as EcoRI-XhoI fragment into the pET28b vector (Novagen, Merek KGaA,
Darmstadt, Germany) to create expression plasmid pRab7bHis. The amino acid sequence of the recombinant Rab7b was identical to that of the native protein cloned by us.\(^5\) Substitution T208A was introduced in PCR reactions using primers: 5'-CCAAACAGGGTG-TGTGTTG and CCGTGTGTTGAGATCTCTTGTG. Phusion DNA polymerase was used in the reactions amplifying whole recombinant plasmid. The reaction mixtures were treated with DpnI endonuclease to remove template DNA and then the 5’ ends of the PCR products were phosphorylated with T4 polynucleotide kinase and circularized with T4 DNA ligase. The recombinant plasmids were verified by sequencing.

Expression and purification of recombinant Rab7b proteins

The recombinant plasmids were introduced into E.coli BL21 (DE3) strain by transformation. Recombinant proteins were produced in mid-logarithmic cultures (OD\(_{600}\) >0.6) in LB medium with kanamycin (50 \(\mu\)g/mL) by induction for 5 h with 1 mM isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG). Cultures were centrifuged, washed with STE buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-Cl pH 8.0), resuspended in buffer B (50 mM HEPES pH 8.0, 0.8 mM CaCl\(_2\), 0.4 mM MgCl\(_2\), pH 7.0, as described by Soldo et al.\(^6\)) and left in MSS for 30 min at 16°C. After spinning down the cell homogenate was obtained as previously described.\(^4\)

Electroporation

P. octoarelia culture prepared as above was resuspended in a fresh volume (100 mL) of MSS buffer for 60 min to prevent autofluorescence.\(^12\) After spinning down and another wash cell viability was examined under a binocular microscope. The buffer G in which recombinant proteins were eluted from the affinity gel was exchanged as follows: 0.5 mL of Sephadex G-25 was loaded on the Spin-X centrifuge tube filters (0.45 µm, Costar Corning) and rinsed fivefold with 0.3 mL of MSS buffer followed by 1 min spin (1000 x g). Recombinant protein solution (130 µL) was overlaid on the column and spun for 4 min. Purified recombinant protein in MSS (50 µg in 50 µL) was mixed with 250 µL of Paramecium suspension (64,000 cells) in a 0.4 mm gap cuvette and electrophoration was performed in a Gene Pulser II Electroporator (Bio-Rad) at the settings: 45 V, 1.5 kV, 0.5 ms at 23°C. Next purification of proteins was performed as above followed by SDS-PAGE, Con A overlay assay as previously described,\(^4\) and Western blotting.

In vitro glycosylation of Rab7b variants

Control experiments were performed before quantification of \([^{14}C]\)UDP-glucose incorporation. Recombinant proteins and cell homogenate at 1:5 ratio were incubated in 50 mM Tris-HCl pH 7.4, 20 mM UDP-glucose (Abcam, Cambridge, UK) and 1 mM EGTA for 30 min at 23°C. Next purification of proteins was performed as above followed by SDS-PAGE, Con A overlay assay as previously described,\(^4\) and Western blotting.

Incorporation of \(\gamma\)-[\(^{32}\)P] ATP by recombinant proteins

The reaction mixture comprised 50 mM Tris-HCl, pH 7.4 and 10 \(\mu\)Ci \(\gamma\)-[\(^{32}\)P] ATP (Perkin Elmer), cell homogene and recombinant protein and incubation was performed for 30 min at 23°C. Aliquots of 10 µL of reaction mixtures were spotted onto PS1 (Whatman) filters that were then washed 3x with 0.75% ortho-phosphoric acid followed by 95% ethanol,\(^13\) air dried and quantified in a scintillation counter (Beckman LS 6500). After adding 5 mL of the ULTIMA Gold scintillator to each filter the second counting was performed immediately. Mean values of radioactivity incorporation were calculated from 3 series of experiments.

In the quantitative studies on \([^{32}\)P] and \([^{14}\)C] incorporation described above the ratio of the volume of His-Select® Nickel Affinity Gel to that of the reaction mixture was strictly controlled in each series of experiments. The efficiency of protein purification was followed by SDS-PAGE/Western blotting with anti-His and specific anti-peptide Rab7b antibody.

Western blotting

SDS electrophoresis and blotting was performed as previously described.\(^14\) After Ponceau S staining detection of phosphoryl-
ed proteins was performed in 2 x PBS, 0.5% BSA, 1% PVP-10 (polyvinylpyrrolidone), PhosSTOP (Roche Diagnostic, Mannheim, Germany), 1% PEG 3500, 0.2% Tween 20) using anti p-Tyr (rabbit, P11230; BD Biosciences, St. José, CA, USA; 1:100) or anti p-Ser (mouse, ALX-804-166-C100; Enzo Life Sciences, Farmingdale, NY, USA; 1:1000) followed by the respective secondary Ab; anti rabbit (1:4000) or anti mouse (1:1000). The protein bands were visualized by an ECL (Amersham Biosciences Hyperfilm ECL, GE Healthcare, Little Chalfont, UK). The film was scanned digitally with a Perfection 2450 Photo Scanner (Epson) and densitometry was performed using GeneTools software (Syngene). Images were processed using Adobe Photoshop CS4 and Illustrator CS4 (Adobe). Omitting of the primary Abs produced no bands.

Stripping was performed after each detection described above with Restore Plus Western Blot Stripping Buffer (Thermo Scientific) for 15 min and followed by washing with PBS containing 0.05% Tween (PBST). Next immunoblotting was done with a specific antipeptide Ab for Rab7b (1:600) in 4% milk in PBST followed by anti-rabbit Ab (1:4000). After subsequent stripping His-tagged proteins were revealed by incubation with monoclonal anti-polyHistidine Peroxidase Conjugate Clone 405 (Promega, Madison, WI, USA) at pH=7.8 (500 pmol/μl). Lys-C, endoproteinase in 50 mM NH4HCO3 buffer of varying pH: Trypsin-Gold, mass spectrometry grade (Promega) at pH=8.0 (1.2 L) and endoproteinase Lys-C, fmol/μl (Promega, Madison, WI, USA) at pH=7.8 (500 pmol/μl). Lys-C, endoproteinase in 50 mM NH4HCO3 buffer of varying 

Proteomic analysis

In-gel digestions was performed as previously described,10 using one of the following enzymes in 50 mM NH4HCO3 buffer of varying pH: Trypsin-Gold, mass spectrometry grade (Promega, Madison, WI, USA) at pH=7.8 (500 pmol/μl), endoproteinase Asp-N from P. fragi (rabbit, P11230; BD Biosciences, St. José, CA, USA; 1:1000) or anti p-Tyr (mouse, ALX-804-166-C100; Enzo Life Sciences, Farmingdale, NY, USA; 1:1000) followed by the respective secondary Ab; anti rabbit (1:4000) or anti mouse (1:1000). The protein bands were visualized by an ECL (Amersham Biosciences Hyperfilm ECL, GE Healthcare, Little Chalfont, UK). The film was scanned digitally with a Perfection 2450 Photo Scanner (Epson) and densitometry was performed using GeneTools software (Syngene). Images were processed using Adobe Photoshop CS4 and Illustrator CS4 (Adobe). Omitting of the primary Abs produced no bands.

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Results

Qualitative and quantitative studies on UDP-Glucose incorporation

Mutagenesis of Thr200 (the putative site of glycosylation in Rab7b) to alanine was confirmed by LC-MS/MS. To check whether glycosylation of the recombinant proteins occurs under our experimental conditions the recombinant proteins were incubated with cell homogenate and UDP-glucose followed by SDS PAGE and Con A overlay assay (Figure 1A). Subsequent Western blot analysis with anti-His Ab that was followed by stripping and reaction with anti-Rab7b specific Ab proved that (His)6 Rab7b variants undergo glycosylation (Figure 1A). In vitro assay with [14C]UDP-glucose indicated that its incorporation into (His)6 Rab7b_200 was diminished by 24% relative to the incorporation into control (His)6 Rab7b_200. Addition of 200 μM UDP-glucose to the reaction mixture diminished the incorporation of [14C]UDP-glucose by 34%.

![Figure 1. Recombinant Rab7b variants undergo glycosylation in the presence of Paramecium octaurelia cell homogenate and UDP-glucose or [14C]UDP-glucose. A)](figure1.png)

**Figure 1.** Recombinant Rab7b variants undergo glycosylation in the presence of Paramecium octaurelia cell homogenate and UDP-glucose or [14C]UDP-glucose. A) Recombinant (His)6 Rab7b proteins were exposed to UDP-glucose and cell homogenate as described in Materials and Methods; after electrophoretic separation of the eluted proteins and Ponceau S staining, Con A overlay assay was performed following by stripping and subsequent Western blotting with anti-His antibody and - after next stripping - with specific anti-Rab7b antipeptide antibody; molecular mass marker (kDa) is shown at the left. B) In vitro assay with [14C]UDP-glucose, recombinant variants and Paramecium homogenate; incorporation into (His)6 Rab7b_200 as compared with the control (His)6 Rab7b; densitometry of bands; intensities of the (His)6 Rab7b_200 protein bands of the Coomassie Blue stained SDS PAGE were calculated as relative optical density r, (His),Rab7b as the control sample. Asterisk indicates significant difference from (His),Rab7b at P<0.1, n=4.
Glycosylation analysis by nanoLC-MS/MS

Glycosylation of Thr200 was revealed by nanoLC-MS/MS analyses of the peptides maps derived by digestion of recombinant Rab7b constructs with endoproteinase Asp-N that was followed by cleavage with trypsin as described in Materials and Methods. After careful comparison differences like those exemplary shown in Figure 2 were evident: an additional peptide ion at m/z=677.6+ was clearly detected in the (His)_6Rab7b recombinant protein that was glycosylated with UDP-glucose (Figure 2A, arrow) which was absent in the control (His)_6Rab7b sample incubated without UDP-glucose in the same experiment (Figure 2B).

To confirm this result, glycosylated (His)_6Rab7b samples were deglycosylated as described in Materials and Methods and again subjected to MS analysis. The peptide ion at m/z=677.6+ was no longer detectable. The fragmentation spectrum of the 677.6+ ion (Figure 2) was verified manually to check for the presence of fragment ions corresponding to the amino acid sequence of the Rab7b protein. As shown in Figure 3, the MSMS spectrum reveals b- and y-ion series of the [195-206] peptide and an additional set of fragment ions representing hybrids of the peptide and a glycan group. The molecular mass of the glycan group was calculated at 624.2 Da based on the difference between the mass of the detected peptide (2029.8 Da) and the calculated mass of non-glycosylated [195-206] peptide with carbamidomethylated cysteine residues equal to 1405.6 Da.

GlycoMod software (http://web.expasy.org/glycomod)18,19 was used for estimation of the nature of the glycan groups attached to the analyzed peptides. As concerns the adduct of 624.1 Da attached to Thr200 the most likely glycan modifications are the following:

i) (Hex)1 (HexNAc)1 (Sulph)3;
ii) (Hex)1 (HexNAc)1 (Phos)3;
iii) (HexNAc)1 (Deoxyhexose)1 (Sulph)1 (HexA)1;
iv) (HexNAc)1 (Deoxyhexose)1 (Phos)1 (HexA)1.

The following phosphoglycosylations are the most likely: (Hex)1(HexNAc)1(Phos)3 or (HexNAc)1(Deoxyhexose)1(Phos)1(HexA)1, with a high preference for the presence of three phosphate groups as in the former case. This conclusion is based on the results indicating a significant decrease in the incorporation of both the [14C]UDP-glucose (Figure 1B) and [32P] into Rab7b_200 (Figure 4B) as compared with the control Rab7b. Three other detected glycosylations of Rab7b at Ser101, Thr131 (and most probably at Ser138) that do not differentiate Rab7a from Rab7b were also found using the same approach.

Figure 2. Comparison between averaged spectra from the nanoLC-MS separation of glycosylated (His)_6Rab7b after UDP-glucose incorporation as described in Materials and Methods (A) with the control recombinant (His)_6 Rab7b incubated without UDP-glucose under the same experimental conditions (B). There is the peptide ion at a mass to charge (m/z) ratio of = 677.6 in the spectrum (A, red arrow) not detected after deglycosylation (B). Inset in (A) shows a blow-up of this peptide ion. Isotopic pattern allows for estimation of ionization ratio as 3+. Both averaged spectra obtained by the accumulation of ca. 40 scans acquired within retention time: 21.4-22.2 min.

Figure 3. MS/MS spectrum derived after fragmentation of the 677.63+ ion detected in glycosylated Rab7b shown in Figure 2. Blue ions: come directly from the spectrum, red ions are derived after deconvolution of the blue ions; green ions: most likely represent hybrids of glycan modification and peptide fragments. In the upper part of the spectrum b-ions and y-ions are marked with corresponding amino acid residues. Additionally, identified sequence of the [195-206] peptide is shown: identification of the exact amino acid residues was based on y-ions (blue residue), b-ions (red residues) or both series (green residues). Black font indicates the single unidentified residue.
**In vitro phosphorylation**

We have previously reported the presence of 8 phosphorylated amino acids in endogenous Rab7a and Rab7b proteins, and proved now that recombinant Rab7b constructs undergo *in vitro* phosphorylation using antibodies against phosphorylated amino acids (Figure 5).

As shown in Figure 4B incorporation of [32P] was significantly decreased by 37% in the case of (His), Rab7b_200 in comparison to that of the control (His), Rab7b.

**Analysis of phosphorylation by nano LC-MS/MS**

Taking the advantage of the recombinant Rab7 variants undergoing *in vitro* phosphorylation as shown in Figures 4 and 5, additional proteomic analyzes were carried out in an attempt to identify all the amino acid residues undergoing this PTM. Figure 6 shows comparison of the phosphorylation patterns of Rab7a and Rab7b.

There are altogether 18 phosphorylated residues in Rab7b and 16 of them are also phosphorylated in Rab7a. Most of them are located in the Rab-specific motifs [9,49]: 14 in Rab7b (including five serine residues) and 13 in Rab7a. Interestingly, there is no such PTM in Rab7a in the RabSF3, while in Rab7b both Ser98 and Ser101 are phosphorylated (Figure 6). Similarly, in the RabSF4 motif three residues are phosphorylated in Rab7b (Ser176, Thr187 and Thr188) and only one in Rab7a (Thr187). As concerns the conserved motifs involved in binding/coordination of Mg²⁺ with phosphate groups, Thr22 in PM1 is phosphorylated in the both proteins, while Ser17 only in Rab7a.

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**Figure 4.** Incorporation of [32P] into recombinant (His), Rab7b and its mutagenized form (His), Rab7b_200 in the presence of *Paramecium octaurelia* cell homogenate. A) Upper panel, Coomassie stained gel (MW marker at the left); lower panel, autoradiogram. B) Equal volumes of eluted recombinant proteins were immediately analyzed in a scintillation counter and their identical aliquots subjected to SDS PAGE; densitometry of bands; intensities of the (His), Rab7b_200 protein bands were calculated as relative optical density *vs* (His), Rab7b control samples. Asterisk represents a significant difference from the control sample at P<0.01 by Student's *t*-test, n=3.

**Figure 5.** Recombinant (His), Rab7b variants undergo *in vitro* phosphorylation. The same blot analysis using antibodies against phosphorylated amino acids. The experiment was conducted as described in Figure 1A. After electrophoretic separation of eluted affinity purified proteins by 15% SDS PAGE and Ponceau Red staining, Western blotting was performed sequentially with anti P-Tyr, anti P-Ser, anti His-tag and anti-Rab7b specific antipeptide Ab. Stripping at each step was performed as described in Materials and Methods. Molecular mass marker is shown at the left.

**Figure 6.** Phosphorylation patterns of *Paramecium octaurelia* Rab7a and Rab7b proteins detected by nano LC-MS/MS.

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F motifs
SF motifs
Green - PM and G motifs
Blue - phosphorylation *out of the motifs*
Red - phosphorylation *within the motifs*
The following phosphorylations were detected beyond the Rab specific motifs: Thr192 in both the proteins and three in Rab7b (Ser95, Thr161, Thr190), whereas in Rab7a: Ser3 and Thr131. Interestingly, the following phosphorylation sites are in agreement with those predicted in Disphos 1.3 data base (http://www.dabi.temple.edu/disphos): in the case of Rab7b: Thr131, Ser176 and Thr188 (both in SF4), whereas in Rab7a only Thr131.

**In vivo electroporation of Paramecium cells to deliver recombinant proteins**

In order to examine intracellular targeting of recombinant (His)_{6}Rab7b proteins *Paramecium octaurelia* cells were electroporated in their presence under strictly controlled conditions as described in Materials and Methods. Double immunodetection under STED confocal microscopy with anti-His and anti-tubulin Abs revealed a proper localization of control recombinant (His),Rab7b to the cytostome area (Figure 7A) and mistargeting of (His),Rab7b_{200} (Figure 7B), the deposits of which were seen in the cells. The two consecutive confocal optical slices shown in Figure 7 C-D clearly reveal the presence of recombinant control Rab7b at the edge of the cytostome, as shown earlier for endogenous Rab7b (compare Figure 6C in Osińska et al.4).

Under a higher magnification tubulin association with the incorporated recombinant (His),Rab7b within the cytostome is visible in the cell (Figure 7E) in a pattern similar to that found for endogenous Rab7b in *Paramecium* (compare Figure 6 E in Osińska et al.4).

**Discussion**

Rab proteins involved in vesicular trafficking appeared early in evolution concomitantly with endomembranes, organelles and cell pathways evolution. They are involved in endocytosis, secretion, cell growth and differentiation,2,19,20 and their paralogues have been reported.6,7,27 Recent data indicate that diverse post-translational modifications could modify the functions of Rab proteins,2,26,28,29 and we found that PTM of Thr200 located in the hypervariable C-terminal region of *Paramecium octaurelia* Rab7b determines its proper intracellular targeting and function.

The presence of O-linked carbohydrates in Rab7b protein explains the discrepancy between the predicted molecular weight of Rab7b and its observed M, in SDS-PAGE also confirming our previous results from 2D gel electrophoresis that revealed two Rab7b immunoreactive spots at slightly different pI values, one of which was glycosylated, similar to bradykinin receptor B2.24 It has also been shown that glycosylated proteins play a role in communication events.24 Kudlow2 pointed out that glycosylation, abundant and reversible, may evoke changes in protein functioning, whereas alternative O-glycosylation/O-phosphorylation of serine-16 in murine estrogen receptor beta has been shown to alter its stability.23

Recently, it has been shown that Rab7b (a new member of mammalian Rabs, displaying 54% identity to human Rab7a)24 involved in retrograde transport from endosomes to the trans-Golgi network25 and trafficking of several receptors,26 coordinates cytoskeletal organization by influencing myosin light chain phosphorylation.27 Our previous results clearly demonstrated that Rab7b was mapped to a cytoskeleton of the structure described as cytostome in *Paramecium* as seen in Figure 6D in Osińska et al.,5 while the present study shows that point mutagenesis at Thr 200 preventing PTM abolishes this targeting.

According to Amoutzias and co-workers,28

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**Figure 7.** In vivo incorporation of recombinant proteins (His),Rab7b and (His),Rab7b_{200} into electroporated *Paramecium octaurelia* cells. Immunolocalization in a STED confocal microscope. Recombinant proteins detected with anti-His antibody conjugated with rhodamine (red), detection of tubulin with antibody conjugated with FITC (green). A) (His),Rab7b, incorporation of the recombinant protein within the cytostome area (arrow). B) (His),Rab7b_{200}; please note dispersed incorporation of the protein; scale bars: 10 µm. C,D,E) (His),Rab7b, C,D) Two consecutive confocal optical slices (1 µm) showing accumulation of (His),Rab7b at the edge of the cytostome (white arrowhead); scale bars: 10 µm. E) Tubulin association with the incorporated control recombinant (His),Rab7b protein within the cytostome is visible at higher magnification (white arrow); scale bars: 5 µm.
Phosphorylation of many Rab proteins has been reviewed by Bucci and Chiarello. Using a large variety of methods they found that in three Rab5 isoforms a common phosphorylation site for Ser/Thr kinases is differentially recognized in vitro by specific kinases. These isoforms are involved in early endocytic events of many markers and PTM could specifically modulate their function in vitro. Furthermore, through interactions with Rab proteins, cargos directly control their own fate: a cargo protein has the ability to interact with more than one Rab and/or with the same Rab in different activation states. In Schizosaccharomyces pombe two Rab7 homologs, Ypl7 and Ypl7, play antagonistic roles in the regulation of vacuolar morphology, whereas phosphorylation of the Rab protein Sec4 has been reported to prevent interactions with its effector and thus controlling exocyst function. Phosphorylation of two amino acid residues in Rab7b that we detected within the RabSF3 motif, one of the regions required for GEF binding may influence such an interaction with an as yet unknown effector.

Thr200 phosphoglycosylation in Paramecium Rab7b was abolished by alanine substitution and could be removed by enzymatic deglycosylation with a mixture of five enzymes of different specificity as was done for nano- LC-MS/MS. Interestingly, tyrosine phosphorylation of Rab24 in cultured mammalian cells HEK293 and HEp-2 was reduced by alanine substitution of two consensus phosphorylation motifs: one in the hypervariable domain (as in the case of Paramecium octaurelia Rab7b) and the other within the motif known as the P-loop (Y17). The latter region is known to influence GTP hydrolysis in Rab proteins, so the phosphorylation of Y17 could contribute to the low intrinsic GTPase activity of Rab24. Recent data show that the hypervariable C-terminal domain (HVD) of Rab7 is required for proper targeting, unlike the HVD of Rab1 and Rab5 that is dispensable for membrane targeting and appears to function simply as a linker between the GTPase domain and the membrane. The N-terminal residues of Rab7 HVD are important for late endosomal/lysosomal localization. We identified here an additional phosphorylation in Paramecium Rab7b in this region at Thr 191, within the Rab SF4 motif, which did not occur in Rab7a (Figure 6). Interestingly, PTM plays also an important role in Paramecium exocytosis: an essential protein in this process is parafusin associated with dense secretory vesicles that undergoes phosphoglycosylation prior to exocytosis and upon its deglycosylation, secretion occurs. Exocytosis is totally blocked upon parafusin down-regulation by RNAi. Recent studies indicate that parafusin is a signaling scaffold protein between nucleus and cilia and emerged early in eukaryotic evolution. We reported previously that expression of Rab7a was 2.6-fold higher than that of Rab7b using qReal-Time PCR analysis. Interestingly, this fact may be related to the recent data on the evolution of two sodium channel paralogues following the teleost-specific WGD, indicating that the Scn4ab gene has a greater propensity toward neofunctionalization due to its decreased expression relative to its paralogue Scn4aa. Moreover, Amoutzias et al.2 analyzing genome and phosphoproteome of Saccharomycyes cerevisiae (which underwent WGD), reported that post-translational regulation impacts the fate of duplicated genes and the number of phosphorylation sites on the proteins they encode is a major determinant of gene retention. In Paramecium octaurelia neofunctionalization of a product of the duplicated Rab7b genes is related to PTM of Thr200 in Rab7b which determines its targeting and putative interaction with bundles of microtubules and structures supporting the oral apparatus. We show that upon mutagenesis of Rab7b this targeting is abolished.

References

1. Axelsen JB, Van KK, Maslov S. Parameters of proteome evolution from histograms of amino-acid sequence identities of paralogueous proteins. Biol Direct 2007;2:32.
2. Amoutzias GD, He Y, Gordon J, Mossiolas D, Oliver SG, Van de Peer Y. Posttranslational regulation impacts the fate of duplicated genes. Proc Natl Acad Sci USA 2010;107:2967-71.
3. Mackiewicz P, Wyroba E. Phylogeny and evolution of Rab7 and Rab9 proteins. BMC Evol Biol 2009;9:101.
4. Osiriska M, Wiejak J, Wypych E, Biliski H, Bartosiewicz R, Wyroba E. Distinct expression, localization and function of two Rab7 proteins encoded by paralogueous genes in a free-living model eukaryote. Acta Biochim Pol 2011;58:397-407.
5. Aury J-M, Jaillon O, Duret L, Noel B, Jubin C, Porcel B, et al. Global trends of whole-genome duplications revealed by the ciliate Paramecium tetraurelia. Nature 2006;444:171-8.
6. Sureda P, Suder P, Wypych E, Biliski H, Bartosiewicz R, Wyroba E. Cloning of two genes encoding Rab7 in Paramecium. Acta Biochim Pol 2006;53:149-56.
7. Li BR, Seabra MC. Targeting of Rab GTPases to cellular membranes. Biochem Soc Trans 2005;33:652-6.
8. Pfeffer SR. Rab GTPase regulation of membrane identity. Curr Opin Cell Biol 2013;25:414-9.
27. Dacks JB, Poon PP, Field MC. Phylogeny of... 1995:65:1290-6.
31. Davis BG, Lloyd RC, Jones JB. Controlled site-selective protein glycosylation for precise glycan-structure-catalytic activity relationships. Bioorg Med Chem 2000:8:1527-35.
32. Rudow JE. Post-translational modification by O-GlcNAc: another way to change protein function. J Cell Biochem 2006:98:1062-75.
33. Cheng X, Hart GW. Alternative O-glycosyla-
34. Zerial M, McBride H. Rab proteins as... 1992:70:715-28.
35. Progida C, Cogli L, Piro F, De Luca A, Mezza X. Rab7b, a novel lysosome-associated small GTPase, is involved in monocytic differentiation of human acute promyelocytic leukemia cells. Biochem Biophys Res Commun 2004:318:792-9.
36. Bucci C, Bakke O, Progida C. Rab7b and receptors trafficking. Commun Integr Biol 2010:3:401-4.
37. Borg M, Bakke O, Progida C. A novel interaction between Rab7b and actinomycin reveals a dual role in intracellular transport and cell migration. J Cell Sci 2014;127:4927-39.
38. Amoutzias GD, He Y, Lilley KS, Van de Peer Y, Oliver SG. Evaluation and properties of the budding yeast phosphoproteome. Mol Cell Proteomics 2012:11:1-13.
39. Barr FA. Review series: Rab GTPases and membrane identity: causal or consequen-
tial? J Cell Biol 2013:202:191-9.
40. Aloisi AL, Bucci C. Rab GTPases-cargo direct interactions: fine modulators of intracellular trafficking. Histol Histopathol 2013:28:839-49.
41. Kashihawaki J, Iwaki T, Takegawa K, Shimoda C, Nakamura T. Two fission yeast rab7 homologs, ypt7 and ypt71, play antago-
nistic roles in the regulation of vacuolar morphology. Traffic 2009:10:912-24.
42. Heger CD, Wrann CD, Collins RN. Phosphorylation provides a negative mode of regulation for the yeast Rab GTPase Sec4p. PLoS One 2011:6:e24332.
43. Day GJ, Mosteller RD, Broek D. Distinct subclasses of small GTPases interact with guanine nucleotide exchange factors in a similar manner. Mol Cell Biol 1998:18; 7444-54.
44. Li F, Yi L, Zhao L, Itzen A, Goody RS, Wu YW. The role of the hypervariable C-terminal domain in Rab GTPases membrane targeting. Proc Natl Acad Sci USA 2014;111: 2572-7.
45. Diekmann Y, Pereira-Leal JB. Bioinformatic approaches to identifying and classifying Rab proteins. Methods Mol Biol. 2015:1298:17-28.
46. Subramanian SV, Satir BH. Carbohydrate cycling in signal transduction: parafusin, a calcium-dependent exocytosis-related phosphoglycoprotein. Proc Natl Acad Sci USA 1994:91:9832-6.
47. Subramanian SV, Satir BH. Carbohydrate... 2015:1298:17-28.
48. Li F, Yi L, Zhao L, Itzen A, Goody RS, Wu YW. The role of the hypervariable C-terminal domain in Rab GTPases membrane targeting. Proc Natl Acad Sci USA 2014;111:2572-7.
49. Liu L, Wyroba E, Christensen ST. Evolutionary implications of localization of the signaling scaffold protein Parafusin to both cilia and the nucleus. Cell Biol Int 2015;39:136-45.
50. Thompson A, Vo D, Comfort C, Zakon HH. Expression evolution facilitated the convergent neofunctionalization of a sodium channel gene. Mol Biol Evol 2014;31:1941-55.