Comparable affinity of RabGDIα for GTP- and GDP-bound forms of Rab7 supports a four-state transition model for Rab7 subcellular localization

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Abstract

Endolysosomal system is linked to almost all aspects of cell life and diseases, and Rab7 occupies a critical node in this crucial pathway. However, there have been conflicting views about the exact role of Rab7 in membrane trafficking, since some studies have reported that Rab7 regulates the trafficking from early to late endosomes, while others highlighted its role in late endosomes to lysosomes progression. In the present study, we have revisited this issue from a new viewpoint. In COS-7 cells, a GDP-bound Rab7 mutant, T22N, was located to vesicular membranes as well as in cytoplasm. Similarly, the GTPase-deficient Q67L mutant of Rab7 resided in cytoplasm as well as on membranes. Additionally, we found that RabGDI interacted with both GTP- and GDP-bound forms of Rab7 *in vitro*. These results have prompted us to propose a four-state transition model for Rab7. This four-state model matches with our recent findings that Rab7 was initially recruited to macropinosomes in a GDP-bound inactive form and subsequently became activated during endocytic maturation in EGF-stimulated COS-7 cells.

Introduction

Endocytic processes are linked to almost all aspects of cell life and diseases. The endocytic transport of proteins and lipids is initiated at the plasma membrane. Endocytic vesicles then fuse with early endosomes (EEs), which mature into late endosomes (LEs) prior to their fusion with lysosomes. By sorting, processing, recycling, storing, activating, silencing, and degrading incoming substances and receptors, endosomes are

responsible for regulation and fine-tuning of numerous pathways in the cell (1,2). Rab5 and Rab7 are of key importance for the endolysosomal system (1,3). Rab5 functions at EEs, whereas Rab7 is required on LEs and lysosomes. Although the role of Rab5 and Rab7 as crucial factors in endolysosomal transport is widely accepted, the coordination of their transition and their multiple interactions on endosomes and lysosomes are far from being fully understood (1,2,4).

Rab7 occupies a critical node in the endolysosomal pathway. It is thought that Rab7 governs the transition from EEs to LEs, and then their trafficking and fusion to lysosomes. However, there have been conflicting views about the exact role of Rab7 in membrane trafficking, since some studies have reported that Rab7 regulates the transition from EEs to LEs (5,6), while others revealed its role in LEs (or autophagosomes) and lysosomes (7-9) progression. In Rab7-depeleted HeLa cells, the existing of cargos, such as EGF-EGFR complex, from the LE/MVB was blocked, resulting in the accumulation of enlarged LEs tightly packed with ILVs (9). This result demonstrated that Rab7 is dispensable for delivery of cargos to the LEs but is required for efficient fusion of the LEs to the lysosomes, at least in EGF/EGFR complex trafficking. However, the restricted role of Rab7 at the timing of LE-lysosome fusion contradicts the broadly-accepted view that Rab7 localizes to both the LEs and lysosomes (10), because it is thought that Rab7-GDP (inactive form) is bound to GDI and thereby resides in the cytoplasm, whereas most researchers assume that the active GTP-form of Rab7 associates onto membranes.

In this study, we have revisited this issue. In COS-7 cells, a GDP-bound Rab7 mutant (Rab7 T22N), was located to vesicular membranes as well as in cytoplasm. On the other hand, the constitutively –active Rab7 Q67L mutant resided in the cytoplasm as

well as on membranes. Furthermore, RabGDI interacted with both GTP- and GDP-bound forms of Rab7 *in vitro*. These results have prompted us to propose the four-state transition model for Rab7 (Fig. 3B). We would like to emphasize that this four-state model matches well with the one of major findings in our recent work 11); i.e., Rab7 was initially recruited to macropinosomes in an GDP-bound inactive form and subsequently became activated during maturation in EGF-stimulated COS-7 cells.

Results

The GDP-bound Rab7 mutant T22N, resided on the vesicular membranes as well as in cytoplasm. We first examined the subcellular distribution of Rab7 T22N in COS-7 cells. According to Fig. 1B in Yasuda et al. (11), most of exogenously-expressed Rab7 was GDP-bound. However, Fig. 1A in this study showed that Rab7 T22N was localized on vesicular structures, probably LEs and lysosomes, as well as in cytoplasm. We hardly detected Rab7 T22N at the cell contour, indicating that no or very little Rab7 T22N was bound to the plasma membrane (Fig. 1A).

The GTP-bound Q67L mutant of Rab7 resided in cytoplasm as well as on membranes. Next, we examined the subcellular distribution of Rab7 Q67L in COS-7 cells (Fig. 1B). According to Fig. 1B in Yasuda et al. (11), the GTP loading of EGFP-Rab7 Q67L used here was 57%. If the two-state transition model (Fig. 3A) is correct, around 57% of EGFP-Rab7 Q67L was in the GTP-bound form and localized to membranes, whereas the remaining (ca. 43%) fraction was GDP-bound and resided in cytoplasm. Our estimation of EGFP-Rab7 Q67L-expressing cells which include that shown in Fig. 1B has suggested a distrust of this prediction. Thus, we tried to test the association of RabGDI with GTP- and GDP-bound forms of Rab7.

RabGDI interacted with both GTP- and GDP-bound forms of Rab7 *in vitro*.

To clarify the nucleotide dependency of the interaction between Rab7 and RabGDI, we performed an *in vitro* binding assay with immobilized GST-RabGDIα and recombinant prenylated Rab7 loaded with GDP or GTPγS. The GTP-bound form of Rab7 bound to RabGDIα with a similar binding activity as Rab7-GDP (Fig. 2). Together with the result shown in Fig. 1B, this binding assay strongly suggests that Rab7-GTP can bind RabGDIα and reside in the cytoplasm.

Discussion

In this study, we propose a four-state transition model for the subcellular localization of Rab7 (Fig. 3), which is based on our findings of the existence of a pool of Rab7-GDP bound to endosomal membranes and a pool of Rab7-GTP present in the cytoplasm. The former existence is supported by three independent evidence. First, Rab7 activity of individual endosomes showed a significant variation different cells at steady-state (Fig. 2 in Yasuda et al. (11)). Second, a fraction of Rab7-T22N, which was practically regarded as Rab7-GDP, because its GTP loading was almost zero (Fig. 1B in Yasuda et al. (11)), was localized to endosomes. Third, during macropinocytosis, Rab7 activity gradually increased after initial recruitment to macropinosomes (Fig. 3 in Yasuda et al. (11)), *In vitro* binding assay showed that GTP-bound form of prenylated Rab7 interacted with RabGDI with a similar affinity as Rab7-GDP did, indicating that Rab7-GTP can exist in the cytosol. For example, the previous experiment with Rab1-D44N, a Rab1 mutant that is unable to bind to RabGDI, has shown that the distribution of Rab1 in cytoplasm is completely dependent on the interaction with RabGDI (12). The possible existence of Rab7-GTP in cytoplasm is supported by the distribution of the FRET/CFP ratio of cytoplasmic Raichu-Rab7 Q67L in several individual COS-7 cells (Kanemitsu-Fujita and Nakamura, unpublished). The distribution of the ratio of Raichu-

Rab7 Q67L, whose GTP loading was 54% (11), , in cytoplasm significantly shifted higher than that of Raichu-Rab7 T22N. This result is explainable by postulating a pool of cytoplasmic Rab7-GTP. Similarly, a weak interaction between Rab5-GDP and RabGDI has been previously reported (13). The comparison between the prevailing and largely accepted two-state model and the four-state model proposed here raises the question on how Rab7 is recruited and activated or inactivated in a living cells. Our previous study (11) has partially addressed this question. Indeed, in EGF-induced macropinocytosis in COS-7 cells, Rab7 was initially recruited to macropinosomes in a GDP-bound form and subsequently become activated by Mon1-Ccz1 during endosome maturation leading to a fusion with lysosomes whose Rab7 activity is independent from Mon1-Ccz1.

In the four-state transition model, the presence of Rab7-GDP on endosomal membranes has enabled us to account for the discrepancies between experimental data and previously-proposed useful but too simplistic “Rab conversion” model (1,14,15). Through a process called Rab conversion, Rab5 on EEs is replaced by Rab7 on LEs/MVBs during early-to-late endosome maturation (5,6). The complex maturation programme of endosomes (from EEs to LEs) is in place to control recycling and other functions of EEs and to allow the progression of LEs within the degradative pathway (1). This Rab conversion has been thought to give unidirectionality to the endocytic process. Recent experiments have shown that Mon1-Ccz1 complex and the CORVET-to-HOPS switch play a role in this process (5,16-18). However, a recent immune-electron microscopy study linking the molecular make-up of endosomes with their ultrastructural characteristics (19) indicates that functionally different intermediates of EEs and LEs can be distinguished, and that the transition between EEs and LEs is rather gradual (20). Furthermore, in phagolysosome formation during apoptosis, the overlap between Rab5 and Rab7 was around 60 min (21). We previously showed that Rab5 and Rab7 reside together on macropinosomes 5-15 min after EGF stimulation of COS7 cells

(Kawasaki *et al.*, unpublished). Thus, there are many evidences for the formation of a hybrid Rab5/Rab7 compartment (19). Furthermore, some researchers have reported that Rab7 regulates the trafficking between LEs and lysosomes (8,9,21), and, at least in some cases, such as EGFR-trafficking in HeLa cells, Rab7 was shown to dispensable for delivery of cargos to the LEs (9). It is very difficult to explain these results directly by simple Rab conversion from EEs to LEs. The present study has demonstrated that some fraction of Rab7-GDP existed on endosomal membranes, and, in EGF-induced macropinocytosis, Rab7 was initially recruited to macropinosomes in a GDP-bound form and subsequently became activated during endosome maturation as described above (11).

We believe that these results provide a new framework which naturally extends beyond the two-state Rab conversion model in case of Rab7; this framework takes into account the formation of a hybrid Rab5/Rab7 endosomal compartment and may explain the discrepancies between the localization of dispensable Rab7 on LEs and the hypothetical absence of inactive Rab7 on endosomal membranes.

How are the results in this study applicable to other situations? We suppose that the behaviour of some Rab GTPases can be better understood based on the four-state transition model. In fact, we have obtained evidences for the existence of Rab35-GDP on membranes and Rab35-GTP in cytosol (Adachi-Ishido *et al*., unpublished). Theoretically speaking, it seems to be rather natural postulating the four-state model for Rab GTPases. It might be fruitful to make efforts to elucidate which Rab population is dominant and which state is relatively minor and transient. It is also unclear the kinetic of Rab7 activation in other types of endocytosis. In clathrin-dependent endocytosis and phagocytosis, Rab7 has been shown to be essential for the fusion of LEs with lysosomes. Thus, it is plausible that a further activation of Rab7 during fusion between LEs and lysosomes is observed in a range of endocytic processes to accelerate fusion. These mechanisms could be cargo- or cell-type specific. Thus, it would be extremely

important to engineer specific biosensors for each member of Rab GTPases and examine their activity-localization relationship in individual cells. This approach would provide fundamental information to understand the basic mechanisms regulating Rab function, and the impact of these small GTPases on a vast range of physiological and pathological processes.

Methods

Plasmids. The cDNAs for wild-type Rab7, Rab7 S22N, and Rab7 Q67L were subcloned into pCAGGS-EGFP (11). The cDNA for wild-type Rab7 was cleaved from a EGFP-Rab7 (22) and subcloned into the pBacPac-His3-TEV2bp vector. pGEX-6P-3-RabGDI has been described previously (23).

Cells, reagents and antibodies. COS-7 cells were maintained in DMEM containing 10% fetal bovine serum. EGF was purchased from Calbiochem. GDP and GTPγS were purchased from Sigma-Aldrich. The following primary antibodies were used: rabbit polyclonal antibodies to Rab7 (Sigma-Aldrich); mouse monoclonal antibody to GST (26H1, Cell Signaling). Anti-GFP polyclonal antibodies were gifts from N. Mochizuki.

Confocal microscopy. Cells were imaged with an IX81 inverted microscope (Olympus) equipped with an FV-1000 confocal imaging system and an automatically programmable XY stage (MD-XY30100T-Meta, SIGMA KOKI) through a plan apochromat 60× oil objective lens (NA 1.4, Olympus).

***In vitro* GST pull-down assay.** 6×His-Rab7 was expressed in Sf9 cells and supplemented with 5 mM mevalonate, a soluble precursor of isopentenylpyrophosphate, which is required for prenylation, in the last 24 h of a 72 h transfection protocol. Cells

were then washed with PBS, sonicated four times in buffer A (10 mM Tris-HCl pH 8.0, 1 mM DTT, 10 mM $MgCl_2$, and protease inhibitors) and centrifuged at 100,000×g for 1 h. The pellet was resuspended in buffer B (20 mM Tris-HCl pH 8.0, 1 mM DTT, 5 mM $MgCl_2$, and 1% CHAPS) and sonicated six times. The suspension was made 300 mM NaCl and centrifuges as before. Purification was performed on NTA-resin (Qiagen) and the recombinant protein was eluted in buffer C (20 mM Tris-HCl pH 8.0, 1 mM DTT, 5 mM $MgCl_2$, 0.6% CHAPS, 500 mM imidazole). The His-tag was then removed by using TEV protease cleavage. GST-RabGDI was prepared as previously described (23). *In vitro* GST pull-down assay were performed as previously described (24) with some modifications. To prepare the GDP-bound and GTPγS-bound forms of 6×His-Rab7, buffer 1 (50 mM Tris-HCl, PH 8.0, 2.5 mM DTT, 25 mM EDTA, and 12.5 mM $MgCl_2$) containing 20 μM GDP or GTPγS and buffer 2 (20 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, and 0.6% CHAPS) containing 6×His-Rab7 were mixed and incubated for 15 min at 30°C. Subsequently, $MgCl_2$ at a final concentration of 10 mM was added to this mixture to stabilize the nucleotides. GDP- or GTPγS-loaded 6×His-Rab7 was incubated with GST-RabGDIα and glutathione Sepharose 4B (GE Healthcare Bioscience) for 1.5 h at 4°C and washed three times buffer 3 (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). Proteins bound to GST- RabGDI were eluted with SDS-sample buffer. The samples were subjected to SDS-PAGE followed by immunoblotting.

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Author contributions

TN, MF, and GS conceived the study; TN AK, SK, and SM designed and performed experiments; TN, MF, and GS analysed and interpreted data; TN wrote the manuscript; all authors discussed the results and commented on the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

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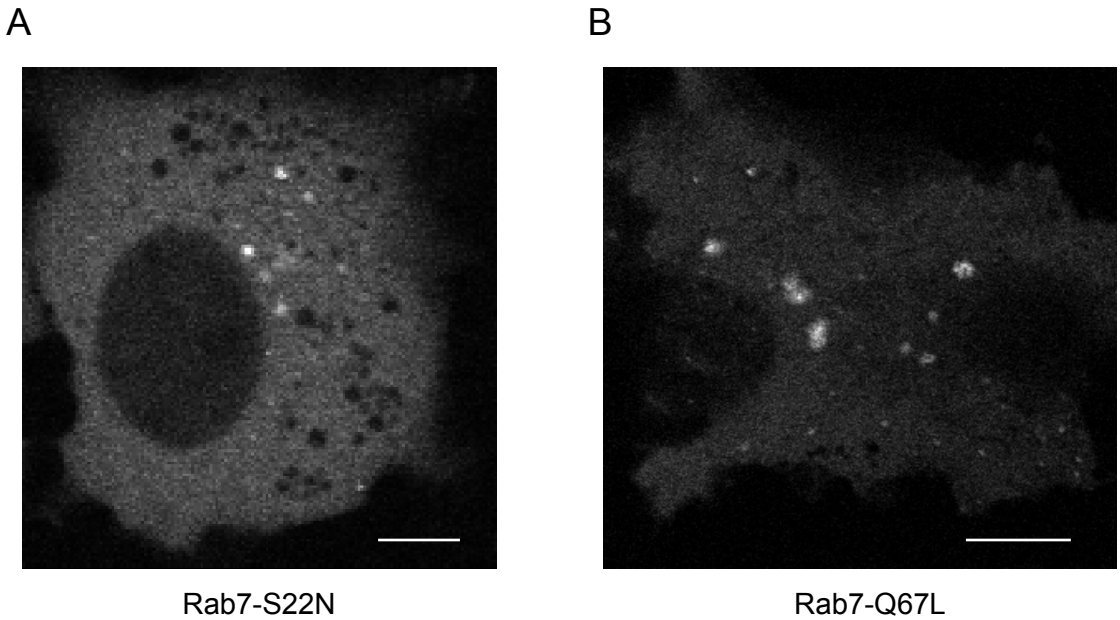
Figure Legends

Fig. 1. Subcellular localization of Rab7 mutants. A representative image of the subcellular distribution of (A) EGFP-Rab7-S22N or (B) EGFP-Rab7-Q67L in COS-7 cells. A bar, 5 μm.

Fig. 2. Affinity of GDP-Rab7 and GTP-Rab7 to RabGDI. The nucleotide dependency was examined by an *in vitro* GST pull-down assay. His-tagged Rab7 prenylated in insect cells and GST-RabGDI was incubated, washed, and then eluted. The eluates were immunoblotted with anti-Rab7 antibodies or anti-GST antibody.

Fig. 3. Four-state transition model for Rab7. (A) In a simple two-state model, the prenylated moiety of Rab7-GDP is masked by RabGDI, and thereby Rab7-GDP resides in cytoplasm. On the contrary, the prenylated moiety of Rab7-GTP is unmasked, and thus Rab7-GTP is localized to endosome membranes. (B) In a four-state transition model proposed here, both GDP-bound and GTP-bound Rab7 can bind to RabGDI and thus reside in cytoplasm. Several experimental evidences have shown the existence of Rab7-GDP on endosome membranes. At present, transitions between respective states are speculative and should be tested experimentally near future.



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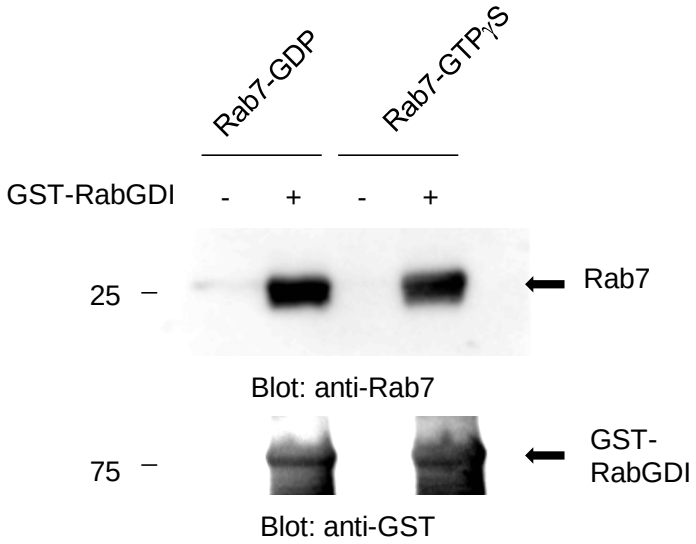

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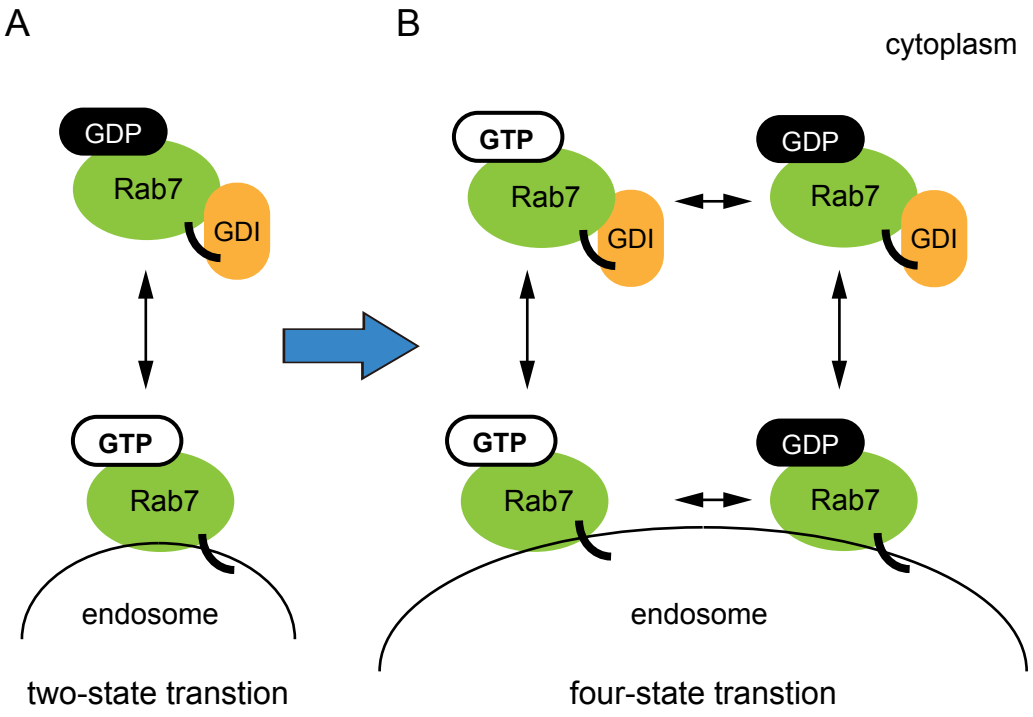

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