Distinct Rab27A binding affinities of Slp2-a and Slac2-a/melanophilin: Hierarchy of Rab27A effectors

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Abstract

The small GTPase Rab27A has recently been shown to regulate melanosome transport in mammalian skin melanocytes through sequentially interacting with two Rab27A effectors, Slac2-a/melanophilin and Slp2-a. Although Slac2-a and Slp2-a contain a similar N-terminal Rab27A-binding domain (named SHD, Slp homology domain), nothing is known about the functional differences between the Slac2-a SHD and Slp2-a SHD. In this study, the Rab27A-binding affinity of ten putative Rab27A effector proteins has been investigated. It has been found that they could be classified into a low-affinity group (e.g., Slac2-a) and a high-affinity group (e.g., Slp2-a and Slp4-a) based on their Rab27A-binding affinity. Kinetic analysis of the GTP-Rab27A-binding to the SHD of Slp2-a, Slp4-a, and Slac2-a by surface plasmon resonance further indicated that the kinetic parameters of Rab27A for the Slp2-a SHD, Slp4-a SHD, and Slac2-a SHD consisted of a fast association rate constant (3.35 × 10^4, 2.06 × 10^4, and 2.11 × 10^4 M⁻¹ s⁻¹, respectively) and a slow dissociation rate constant (4.48 × 10⁻⁴, 3.96 × 10⁻⁴, and 2.37 × 10⁻³ s⁻¹, respectively), and their equilibrium dissociation constants were determined to be 13.4, 19.2, and 112 nM, respectively. Our data suggest that distinct Rab27A binding activities of Slac2-a and Slp2-a ensure the order (or hierarchy) of Rab27A effectors that sequentially function in melanosome transport in melanocytes.

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novel Rab27A-binding domain distinct from the SHD and is a putative priming factor for exocytosis by certain immune cells [24,25].

Slac2-a, the best characterized Rab27A effector present on melanosomes, has been shown to control transfer of melanosomes from microtubules to actin filaments [9,14,19–22,26] as well as actin-based melanosome transport [27] by forming a tripartite protein complex between Rab27A, Slac2-a, and an actin-based motor myosin Va. Another Rab27A effector, Slp2-a, has recently been shown to be present on melanosomes [6] and to regulate a later stage of melanosome transport (i.e., anchoring of melanosomes to the plasma membrane) through interaction with phosphatidylserine in the plasma membrane [14]. These findings have strongly indicated that Rab27A sequentially utilizes two Rab27A effectors (i.e., Slac2-a and Slp2-a) in melanosome transport in melanocytes. Expression of several Rab27A effectors in one secreting cell type has also been reported [8,10,18,28–31], but the functional relationships between them during granule exocytosis have never been elucidated.

If a hierarchy of Rab27A effectors exists in Rab27A-dependent membrane transport, Slp2-a and Slac2-a would be expected to have different Rab27A-binding affinities. It should be noted that the Slp2-a SHD (i.e., type II SHD) and the Slac2-a SHD (i.e., type I SHD) structurally differ in terms of only the latter containing two zinc finger motifs (see Fig. 1A) [3]. Although both the type II SHD (i.e., Slp1, Slp2-a, and Slac2-b) and type I SHD (others) have been shown to bind the GTP-bound form of Rab27A [6,32,33], functional diversity of the SHD (e.g., distinct Rab27A binding activity of the type I SHD and the type II SHD) has never been elucidated.

In this study, I investigated and compared the Rab27A-binding affinity of Slp1–5, Slac2-a–c, rabphilin, and Noc2 and found that these Rab27A-binding proteins can be classified into two groups in terms of their Rab27A-binding affinity, a high-affinity group (i.e., Slp1, Slp2-a, Slp4-a, Slp5, Slac2-b, rabphilin, and Noc2) and a low-affinity group (i.e., Slp3-a, Slac2-a, and Slac2-c). Based on this finding, I discuss the physiological significance of the presence of two Rab27A effectors having distinct Rab27A-binding affinities in relation to membrane trafficking.

**Materials and methods**

**Antibodies.** Anti-T7 tag antibody-conjugated agarose and horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody were from Merck Biosciences Novagen (Darmstadt, Germany). HRP-conjugated anti-GST (glutathione S-transferase) antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG M2 Affinity Gel, HRP-conjugated anti-FLAG M2 mouse monoclonal antibody, and HRP-conjugated anti-HA tag mouse monoclonal antibody were from Sigma Chemical Co. (St. Louis, MO).

**Plasmid construction and site-directed mutagenesis.** The Slp2-a-SHD, Slp4-a-SHD, or Slac2-a-SHD fragment [6] was subcloned into the pEF-T7-GST or pEF-FLAG-GST expression vector (modified from pEF-BOS [19,34–36]), and the resultant vectors were referred to as pEF-T7-GST-Slp2-a, pEF-T7-GST-Slp4-a-SHD, and pEF-FLAG-GST-Slac2-a-SHD, respectively.

Mutant Rab27A containing a Trp-Gly mutation at amino acid position 73 (W73G), L130P, A152P, and C219/221A mutation was constructed by PCR essentially as described previously [37], using the following oligonucleotides with a restriction enzyme site (underlined) or a stop codon (boldface): 5'-CTCAGTTAGGOGACACGCCA-3' (W73G primer, sense), 5'-ACGCGTAGTSSAAGACCATATAGTGGCGT-3' (L130P primer, sense), 5'-CCCCGGGACTCTTTCCGGAAAG-3' (A152P primer, sense), and 5'-TCAAAGGCCAGACCAACCCCTTCT-3' (C219A/C221A primer, antisense). The mutant Rab27A fragments, including Rab27A(W73G/L130P/A152P/C219/221A) fragment [32], were subcloned into the pGEX-4T-3 vector (Amersham Biosciences, Buckinghamshire, UK). The Rab27A(C219/221A) fragment was also subcloned into the pEF-FLAG expression vector [36,38].

Other mammalian expression vectors (pEF-T7-Slp1–5-SHD, pEF-T7-Slp1–5, pEF-T7-Slac2-a–c-SHD, pEF-T7-Slac2-a–c, pEF-T7-rabphilin-RBD (Rab-binding domain), pEF-T7-rabphilin, pEF-T7-Nox2-RBD, pEF-T7-Nox2, pEF-FLAG-Rab27A, and pEF-FLAG-Rab27A(C781L)) were produced as described previously [6,7,10,11,17,19].

**Co-immunoprecipitation assay in COS-7 cells.** COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin, at 37 °C under 5% CO₂, pEF-T7 and/or pEF-FLAG vectors (a total of 4 μg of plasmids) were transfected into COS-7 cells (7.5 × 10⁵ cells, the day before transfection/10 cm-dish) by using LipofectAmine Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s notes. Three days after transfection, cells were harvested and homogenized, and total cell lysates were prepared as described previously [36,39]. The total cell lysates (400 μl) were incubated at 4 °C for 1 h with either anti-T7 tag
antibody-conjugated agarose beads or anti-FLAG M2 Affinity Gel (wet volume 20 μl) with gentle agitation, and the proteins bound to the beads were analyzed by 12.5%, 10%, or 7.5% SDS–PAGE followed by immunoblotting with HRP-conjugated anti-T7 tag antibody (1/10,000 dilution) and HRP-conjugated anti-FLAG M2 antibody (1/10,000 dilution) [36, 40]. The immunoreactive bands were visualized with an enhanced chemiluminescence kit (Amersham Biosciences). The intensity of the bands on X-ray film was quantified with Lane Analyzer (version 3.0) (ATTO Corp., Tokyo, Japan) as described previously [38]. The blots shown in this paper are representative of at least two or three independent experiments. 

GST pull-down assay. GST-Rab27A mutants were expressed in Escherichia coli JM109 and purified by standard protocols. Glutathione-Sepharose beads (wet volume 20 μl; Amersham Biosciences) coupled with 5 μg of the purified GST-Rab27A mutants were incubated for 1 h at 4 °C with 400 μl of COS-7 cell lysates containing T7-Slp1–5, T7-Slac2-a–c, T7-rabphilin, or T7-Noc2 in 50 mM Hepes–KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 0.5 mM GTP/S, 1% Triton X-100, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A). After washing the beads three times with 1 ml of 50 mM Hepes–KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 0.2% Triton X-100, and protease inhibitors, proteins bound to the beads were analyzed by 7.5% SDS–PAGE followed by immunoblotting with HRP-conjugated anti-T7 tag antibody (1/10,000 dilution) and by Amido Black staining.

Surface plasmon resonance (SPR). GST-Slp2-a-SHD and GST-Slp4-a-SHD were transiently expressed in COS-7 cells (more than twenty 10-cm dishes) and affinity-purified with glutathione-Sepharose beads as described above. FLAG-Rab27A(Q78L) and FLAG-GST-Slac2-a-SHD expressed in COS-7 cells were affinity-purified with an anti-FLAG M2 Affinity Gel, and bound proteins were eluted with 0.1 mg/ml FLAG peptide (Sigma Chemical Co.). FLAG peptide in the eluate was removed by dialysis for 8 h against 500 ml (v) of 50 mM Hepes–KOH, pH 7.2, 150 mM NaCl, and 1 mM MgCl2. All these purification procedures were carried out at 4°C to avoid degradation of recombinant proteins. Under these conditions, however, the purified recombinant GST-Slac2-a-SHD protein contained a degradation product (an additional 35 kDa band), probably because Slac2-a contains multiple PEST-like sequences (potential signals for rapid protein degradation) and is easily degraded by proteases [42], while other recombinant proteins were obtained without or little contamination by degradation products. Purified proteins were subjected to 10% SDS–PAGE followed by staining with Coomassie brilliant blue R-250, and protein concentrations were determined on SDS–polyacrylamide gels, using bovine serum albumin as a reference. 

Association and dissociation of GST-SHDs with FLAG-Rab27A(Q78L) were measured with the BIACORE biosensor system 3000 (Biacore, Uppsala, Sweden). Anti-GST antibody (Biacore) was coupled to a carboxymethyl-dextran CM5 sensor chip with an amine coupling kit (Biacore) containing 1 mM MgCl2 and 0.5 mM GTP/S. A 10 mM glycine–HCl (pH 2.2) solution was used to regenerate the chip. Data analysis was performed with BIA evaluation version 4.1 software (Biacore).

Results

Distinct Rab27A-binding affinities of Slps and Slac2s

I previously found that the SHDs of Slp1–5 and Slac2-a–c function as a specific Rab27A/B-binding domain [6] and that their SHDs can be classified into two groups structurally [3]: a type I SHD group that consists of SHD1 and SHD2 separated by two zinc finger motifs, and a type II SHD group that consists of SHD1 and SHD2 alone (Fig. 1A). To determine the functional differences between type I SHDs and type II SHDs, I first investigated the effect of the C-terminal geranylgeranylation [43] on the Rab27A binding activity of three well-characterized Rab27A effector molecules, Slp2-a (type II SHD), Slp4-a (type I SHD), and Slac2-a (type I SHD). To do so, I prepared a geranylgeranylation-deficient mutant of Rab27A by substitutions of Ala for Cys at amino acid positions 219 and 221. The mutant Rab27A(C219/221A) with FLAG tag was found to be
expressed in the cytosol (or soluble fraction) of COS-7 cells because of the lack of geranylgeranylation and failure to associate with the membrane fractions of COS-7 cells (data not shown). As shown in Fig. 1B, the Rab27A(C219/221A) mutant interacted with Slac2-a, Slp2-a, and Slp4-a, the same as the wild-type protein (lanes 4, 6, and 8 in the top panel), indicating that geranylgeranylation of Rab27A is not required for recognition by the SHD. I noted, however, that a much smaller amount of Rab27A bound to Slac2-a than to Slp2-a and Slp4-a, suggesting that the Rab27A-binding affinity of Slac2-a is much lower than that of Slp2-a and Slp4-a. Consistent with this notion, competition experiments clearly showed that Rab27A preferentially interacted with Slp2-a and Slp4-a rather than with Slac2-a (middle panels of Figs. 2A and B, respectively), and that Slac2-a hardly interacted with Rab27A at all even though ten times more Slac2-a protein was present in the reaction mixtures than the Slp2-a and Slp4-a proteins (lane 6 in the middle panels of Figs. 2A and B, respectively).

This finding prompted us to further investigate and compare the relative Rab27A-binding affinity of Slp1–5-SHD, Slac2-a–c-SHD, rabphilin-RBD, and Noc2-RBD by co-immunoprecipitation assay in COS-7 cells. In brief, these ten Rab27A-binding proteins with T7 tag were expressed in COS-7 cells, and their expression levels were evaluated by immunoblotting with anti-T7 tag antibody. Since the expression levels of the recombinant proteins in the total cell lysates varied among the ten Rab27A-binding proteins, cell lysates containing high expression levels of T7-tagged proteins (e.g., type I SHD) were appropriately diluted so that the amounts of T7-tagged proteins in the diluted lysates of the COS-7 cells were similar (Fig. 3, bottom panel). It should be noted that the expression levels of the type II SHD (i.e., Slp1-SHD, Slp2-a-SHD, and Slac2-b-SHD) in COS-7 cells were always very low compared to the type I SHD (Fig. 3), suggesting that the presence of zinc finger motifs between SHD1 and SHD2 may stabilize the structure of SHD in living cells [32]. The resultant COS-7 cell lysates containing a similar amount of T7-tagged proteins were further diluted to 1, 1/4, and 1/10, and the diluted samples were incubated with beads coupled with FLAG-Rab27A to compare the relative Rab27A-binding affinity of ten Rab27A-binding proteins. Proteins bound to the beads were analyzed by immunoblotting with anti-T7 tag antibody (middle panel in Fig. 4A), and the intensity of the immunoreactive bands on X-ray film was quantified (Fig. 4B). I found that ten Rab27A-binding proteins could be classified into two groups in terms of their Rab27A-binding affinity: a high-affinity group (i.e., Slp1, Slp2-a, Slp4-a, Slp5, Slac2-b, rabphilin, and Noc2) and a low-affinity group (i.e., Slp3-a, Slac2-a, and Slac2-c; asterisks in Fig. 4B).

**Kinetic analysis of the interaction between Rab27A and Slp2-a, Slp4-a, and Slac2-a by SPR**

To determine the affinity of Slp2-a, Slp4-a, and Slac2-a for Rab27A in greater detail, T7-GST-Slp2-a-SHD, T7-GST-Slp4-a-SHD, or FLAG-GST-Slac2-a-SHD expressed in COS-7 cells was immobilized on a CM5 sensor chip coupled to anti-GST antibody, and interactions between GST-fusion proteins and purified recombinant Rab27A(Q78L) protein (mimics the GTP-bound form of Rab27A) were analyzed by SPR using Biacore 3000 (see Materials and methods for details) (Fig. 5). Kinetic analysis revealed that the kinetic parameters of Rab27A(Q78L) for GST-Slp2-a-SHD and GST-Slp4-a-SHD consisted of a fast association rate constant ($k_{\text{ass}} = 3.35 \times 10^4$ and $2.06 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively) and a slow dissociation rate constant ($k_{\text{diss}} = 4.48 \times 10^{-4}$ and $3.96 \times 10^{-4}$ s$^{-1}$, respectively), and the equilibrium dissociation constant ($K_D = k_{\text{diss}}/k_{\text{ass}}$) was determined to be 13.4 and 19.2 nM, respectively, in 10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM MgCl$_2$, 0.005% surfactant P20, and 0.5 mM GTPyS. By contrast, however, the observed dose-dependent SPR signals of GST-Slac2-a-SHD were very small (Fig. 5C), and the SPR traces obtained were not well fitted by the 1:1 binding curves ($k_{\text{ass}} = 6 \times 10^4$ M$^{-1}$ s$^{-1}$, $k_{\text{diss}} = 5 \times 10^{-3}$ s$^{-1}$, and $K_D = 80$ nM). Since the purified GST-Slac2-a-SHD contained a degradation product (i.e., an additional 35 kDa band) [42], we further analyzed the SPR traces by the heterogeneous ligand-parallel reaction model and found that they were well fitted by the heterogeneous ligand binding curves (Supplemental Fig. 1). The high-affinity component of Rab27A(Q78L) binding to...
GST-Slac2-a-SHD consisted of $k_{\text{ass1}} = 2.11 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, $k_{\text{diss1}} = 2.37 \times 10^{-3} \text{ s}^{-1}$, and $K_D1 = 112 \text{ nM}$, and the low-affinity component consisted of $k_{\text{ass2}} = 1.17 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, $k_{\text{diss2}} = 0.267 \text{ s}^{-1}$, and $K_D2 = 2.28 \text{ lM}$. The low-affinity Rab27A binding activity of Slac2-a, Slac2-c, and Slp3-a was most likely to be mediated by the degradation product of Slac2-a-SHD. Taken together, we concluded that Slac2-a interacts with Rab27A with much lower affinity than Slp2-a (or Slp4-a) does.

**Missense mutations of Rab27A found in Griscelli syndrome patients affect the binding of effector molecules differently**

Since the Rab27A-binding affinities of Slp2-a and Slac2-a, two Rab27A effectors that function in melanosome transport [14], were different, finally I used a GST pull-down assay to investigate whether the missense mutations of Rab27A found in type II Griscelli syndrome patients [44–46] affect the binding of these effector molecules differently. As shown in **Fig. 6**, all of the missense mutations (i.e., W73G, L130P, and A152P) found in type II Griscelli syndrome patients and the mutations in the switch II region of Rab27A (i.e., L84I/F88Y/D91G) [32] completely abrogated the binding of Slac2-a, consistent with previous reports [32,45,46]. Slp2-a, however, bound the Rab27A(W73G) and Rab27A(L84I/F88Y/D91G) mutants with reduced binding activity, and did not bind the Rab27A(L130P) and Rab27A(A152P) mutants at all. Similar experiments were performed for other Rab27A-binding proteins, and the results showed that the low-affinity group of Rab27A-binding proteins did not interact with any of the Rab27A mutants at all, whereas the high-affinity group of Rab27A-binding proteins interacted differently with the W73G mutant and L84I/F88Y/
D91G Rab27A mutant, but mostly abrogated binding activity toward the L130P mutant or A152P mutant. For example, Slp1, Slp2-a, Slp4-a, and rabphilin bound both the Rab27A(W73G) mutant and Rab27A(L84I/F88Y/D91G) mutant, Noc2 bound the Rab27A(L84I/F88Y/D91G) mutant, and Slp5 did not interact with any of the Rab27A mutants, the same as the low-affinity group. These results suggest that the missense mutations of Rab27A found in human type II Griscelli syndrome primarily affect the function of the low-affinity group of Rab27A effectors (Slp3-a, Slac2-a, and Slac2-c), Slp5, and Slac2-b. In fact, melanocytes from type II Griscelli syndrome patients with Rab27A missense mutations exhibit the perinuclear aggregation phenotype (=dysfunction of the low-affinity type Slac2-a), not the peripheral dilution phenotype (=dysfunction of the high-affinity type Slp2-a) [14,44–46].

Discussion

In the present study, I demonstrated that the Rab27A-binding domains of Slp1–5, Slac2-a–c, rabphilin, and Noc2 can be classified into two groups in terms of their Rab27A-binding affinity (Figs. 4 and 5): a low-affinity group, consisting of Slp3-a, Slac2-a, and Slac2-c, and a high-affinity group, consisting of Slp1, Slp2-a, Slp4-a, Slp5, Slac2-b, rabphilin, and Noc2. This finding makes it possible to explain why the endogenous Slp2-a·Rab27A complex in melanocytes (or Slp4-a·Rab27A/B complex in secreting cells) is always more efficiently immunoprecipitated from the cell lysates than the Slac2-a·Rab27A complex (or Slac2-c·Rab27A/B complex) [14,28,31]: the Slac2-a of the Slac2-a·Rab27A complex is readily replaced by Slp2-a to form a stable Slp2-a·Rab27A complex during the course of immunoprecipitation. I have previously demonstrated that the presence of zinc finger motifs between SHD1 and SHD2 has no relation to Rab27A-binding affinity, although the zinc finger motifs seemed to be important for stable expression of the entire SHD in living cells (Fig. 3) [32]. Kinetic analysis of the three representative Rab27A effector molecules, Slp2-a, Slp4-a, and Slac2-a, revealed that they rapidly bind Rab27A ($k_{ass} = \sim 2 \times 10^4 M^{-1} s^{-1}$), but that the dissociation process is very slow ($k_{diss} = \sim 4 \times 10^{-4} s^{-1}$ for Slp2-a, Slp4-a; and $\sim 2 \times 10^{-3}$ for Slac2-a) (Fig. 5). The propensity for rapid association by Rab27A with the SHD and slow dissociation by Rab27A from the SHD, together with the low intrinsic GTPase activity of Rab27A[47], is well adapted to the function of Slac2-a as a myosin Va receptor during melanosome transport [9,19–22,26] and the function of Slp2-a that anchors melanosomes to the plasma membrane [14].

It is interesting to note that the W73G mutation or L84I/F88Y/D91G mutation in Rab27A affected recognition by ten Rab27A-binding proteins differently, whereas the L130P mutation or A152P mutation in Rab27A dramatically reduced (or mostly abrogated) binding activity toward all Rab27A-binding proteins tested (Fig. 6). The latter finding is quite consistent with the previous findings that neither Rab27A(L130P) nor Rab27A(A152P) binds GTP or GDP in vitro[45] and that SHD is a GTP-dependent Rab27A-binding motif [6,32]. The former mutations are likely to affect the protein interaction itself, rather than GTP/GDP-binding, because Rab27A(W73G) has been shown to bind GTP [45,46] and Rab27A(L84I/F88Y/D91G) has been shown to bind rabphilin normally [32]. The SHD1 of Slp1–5 and Slac2-a–c presumably binds the same switch II region (amino acid residues 77–93) of Rab27A (i.e., competition between Slp2-a and Slac2-a or between Slp4-a and Slac2-a occurs; Fig. 2) [32], but it may recognize different amino acid residues in the switch II region of Rab27A. Since the amino acid identity scores of the SHD of Slp and Slac2 and the RBD of rabphilin and Noc2 are not very high [48], I am unable to identify the amino acids in SHD that are responsible for the high- or low-affinity Rab27A-binding activity. A structural analysis (e.g., three-dimensional...
structure of Rab27A-SHD complex) will be necessary to resolve this issue.

Although the expression and localization of Slp3-a protein have never been elucidated, I noted that one of two low-affinity type Rab27A effectors, Slac2-a and Slac2-c, is always co-expressed with one of the high-affinity type Rab27A effectors. For example, Slac2-a is co-expressed with Slp2-a in melanocytes [6,14], and Slac2-c is co-expressed with Slp4-a [8,28–30], Noc2 [10,18], and/or rabphilin [10] in certain endocrine cells, and with Slp4-a in parotid acinar cells [31]. Since Slac2-a and Slp2-a sequentially function in Rab27A-dependent melanosome transport (i.e., low-affinity type Slac2-a functions in the early stage of melanosome transport and high-affinity type Slp2-a in the late stage) [14], it is highly possible that two Rab27A effectors sequentially function in the transport of secretory granules in secretory cells (i.e., low-affinity type Slac2-c functions in the early stage of granule transport, and high-affinity type Slp4-a or rabphilin in the late stage, presumably the docking step of dense-core vesicle exocytosis [49,50]). The hierarchy of Rab27A effectors in granule transport (or granule exocytosis) in PC12 cells or parotid acinar cells is now under investigation in my laboratory.

In summary, I have demonstrated that Rab27A-binding proteins, Slp1–5, Slac2-a–c, rabphilin, and Noc2, can be classified into two groups in terms of their Rab27A binding activity (a low-affinity group, Slp3-a, Slac2-a, and Slac2-c; and a high-affinity group, Slp1, Slp2-a, Slp4-a, Slp5, Slac2-b, rabphilin, and Noc2). This finding suggests that their distinct Rab27A binding activities ensure the order (or hierarchy) of Rab27A effectors that sequentially function in Rab27A-dependent organelle transport.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.03.001.

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