Rabphilin dissociated from Rab3 promotes endocytosis through interaction with Rabaptin-5

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SUMMARY

Rabphilin is a secretory vesicle protein that interacts with the GTP-bound form of the small GTPase Rab3. We investigated the involvement of Rabphilin in endocytosis using different point mutants of the protein. Overexpression of wild-type Rabphilin in the insulin-secreting cell line HIT-T15 did not affect receptor-mediated transferrin endocytosis. By contrast, Rabphilin V61A, a mutant that is unable to interact with Rab3, enhanced the rate of transferrin internalization. The effect of Rabphilin V61A was not mimicked by Rabphilin L83A, another mutant with impaired Rab3 binding. Careful analysis of the properties of the two mutants revealed that Rabphilin V61A and Rabphilin L83A are both targeted to secretory vesicles, have stimulatory activity on exocytosis, and bind equally well to α-actinin. However, Rabphilin L83A fails to interact with Rabaptin-5, an important component of the endocytic machinery. These results indicate that Rabphilin promotes receptor-mediated endocytosis and that its action is negatively modulated by Rab3. We propose that the hydrolysis of GTP that is coupled to the exocytotic event disrupts the Rabphilin-Rab3 complex and permits the recruitment of Rabaptin-5 at the fusion site. Our data show that immediately after internalization the transferrin receptor and VAMP-2 colocalize on the same vesicular structures, suggesting that Rabphilin favors the rapid recycling of the components of the secretory vesicle.

Key words: GTP, Recycling, Exocytosis

INTRODUCTION

Tight coupling between exocytosis and endocytosis is required to prevent excessive enlargement of the plasma membrane and to permit rapid recycling of the components of the secretory vesicle. This is most dramatically demonstrated in neurons, where impairment of endocytosis leads to rapid depletion of the secretory vesicle pool (Koenig and Ikeda, 1989). The mechanism ensuring the spatial and temporal link between exo- and endocytosis is not well understood. The observation that synaptotagmin binds with high affinity to the clathrin adaptor complex AP-2 led to the proposal that the coupling between the two processes could be provided by proteins associated with the membrane of secretory vesicles (Zhang et al., 1994). According to this model, the insertion of these proteins in the plasma membrane would provide the signal that triggers endocytosis. Multidomain adaptor molecules such as intersectin that bind concomitantly to components of the exocytotic and endocytotic machinery (Okamoto et al., 1999) could contribute an additional connection between the two pathways.

The four isoforms of the small GTPase Rab3 modulate exocytosis in most secretory systems, including neurons and exocrine and endocrine cells (Lledo et al., 1993; Holz et al., 1994; Johannes et al., 1994; Geppert et al., 1997; Ohnishi et al., 1997; Iezzi et al., 1999). Rab3 undergoes a functional cycle that is coupled to GTP hydrolysis. In the GDP-bound form, Rab3 forms a cytosolic complex with the regulatory protein Rab GDP-dissociation inhibitor (RabGDI) (Regazzi et al., 1992, Ullrich et al., 1993). RabGDI prevents the binding of Rab3 to inappropriate cellular compartments and delivers the protein to the secretory vesicle membrane (Pfeffer et al., 1995). There, through interaction with the guanine nucleotide exchange factor Rab3-GEP (Wada et al., 1997), Rab3 substitutes GDP for GTP. This causes a conformational change that allows interaction with specific effector proteins. During or immediately after fusion of the secretory vesicle with the plasma membrane, a regulatory protein called Rab3-GAP enhances the intrinsic enzymatic activity of Rab3 and promotes the hydrolysis of GTP (Fukui et al., 1997). Once this has occurred, GDP-bound Rab3 reforms the complex with RabGDI and dissociates from the membrane.

Rabphilin is a 78 kDa protein purified by chemical crosslinking of Rab3 with bovine brain extracts (Shirataki et al., 1992). Rabphilin is associated with the membrane of secretory vesicles and interacts selectively with the GTP-bound form of Rab3 (Shirataki et al., 1993; Stahl et al., 1996). Structural and biochemical analyses indicate that Rabphilin can be subdivided into two functionally distinct domains (Yamaguchi et al., 1993; Ostermeier and Brunger, 1999). The N-terminal portion contains an amphipathic α-helix required for Rab3 binding, whereas the C-terminal region possesses two C2-like domains that bind Ca2+ and phospholipids. Several observations indicate that Rabphilin performs major regulatory
functions in exocytosis. Overexpression of the full-length protein stimulates exocytosis in chromaffin and insulin-secreting cells (Chung et al., 1995; Arribas et al., 1997; Joberty et al., 1999). By contrast, fragments of Rabphilin lacking one or both C2 domains act as potent dominant inhibitors of secretion (Chung et al., 1995). In addition, microinjection of the isolated N- or C-terminal parts of Rabphilin inhibits Ca2+-triggered cortical granule exocytosis in mouse eggs (Masumoto et al., 1998). The precise mechanism of action of Rabphilin in exocytosis is still unclear. The protein was initially postulated to mediate Rab3 action. However, more recent data clearly indicate that Rab3 and Rabphilin can modulate secretion independently. Thus, mutants of Rabphilin that do not interact efficiently with Rab3 can still potentiate stimulus-induced secretion (Joberty et al., 1999). Conversely, mutants of Rab3 that cannot bind to Rabphilin inhibit exocytosis (Chung et al., 1999; Coppola et al., 1999). These observations are further substantiated by the fact that the synaptic properties that are impaired in Rab3A-deficient mice are not altered in Rabphilin knock-out mice (Schütter et al., 1999). A yeast two-hybrid screen revealed that the N-terminal portion of Rabphilin can bind to α-actinin (Kato et al., 1996). The binding of Rabphilin enhances the actin filament bundling activity of α-actinin (Kato et al., 1996). Interestingly, the GTP-bound form of Rab3 competes for the binding of α-actinin (Kato et al., 1996). These findings suggest that at least part of the function of Rabphilin is mediated by the reorganization of actin filaments.

In addition to its effect on exocytosis, Rabphilin has been suggested to participate in the control of endocytosis. Thus, the membrane area of endosomes is increased following injection of full-length Rabphilin in squid giant synapse, whereas injection of the N-terminal fragment prevents membrane retrieval from the plasma membrane (Burns et al., 1998). Yeast two-hybrid screening showed that Rabphilin can bind to Rabaptin-5, a protein that associates with the GTP-bound form of Rab5 (Ohya et al., 1998). The interaction with Rabaptin-5 occurs through the N-terminal fragment of Rabphilin and is prevented by the presence of the GTP-bound form of Rab3 (Ohya et al., 1998).

In this study, we took advantage of different point mutants of Rabphilin to investigate the role of this protein in endocytosis. We found that a Rabphilin mutant that is unable to bind Rab3 can promote transferrin endocytosis by a mechanism that requires interaction with Rabaptin-5. We propose that Rabphilin plays a dual role in vesicular trafficking at the plasma membrane by participating in the regulation of distinct stages of the exocytic and endocytic pathways.

MATERIALS AND METHODS

The generation of Rabphilin mutants has been described previously (Joberty et al., 1999). Human transferrin receptor cDNA obtained from L. Kuhn (Swiss Cancer Research Institute, Epalinges) was subcloned in pCDNA3 (Invitrogen). Epitope-tagged full-length human Rabaptin-5 in pCDNA3 was kindly provided by G. Greninger (University of Lausanne). The 385-753 fragment of Rabaptin-5 amplified by PCR from the full-length cDNA was subcloned in pCDNA3. The VAMP-2-Tag plasmid and the monoclonal antibody (KT3) against the T antigen tag were obtained from R. B. Kelly (University of California, San Francisco). Human transferrin (hTfr) coupled to FITC was from Molecular Probes; hTfr coupled to horseradish peroxidase (HRP) was purchased from Pierce. The Cy3-conjugated anti-mouse antibody was from Jackson Immuno Research Laboratories. Purified chicken gizzard α-actinin and the antibody against α-actinin were obtained from Sigma.

Cell culture and transfection

HIT-T15 cells were cultured in RPMI 1640 supplemented with 5% fetal calf serum as described previously (Regazzi et al., 1990). Transient cotransfection was performed by electroporating 3×10^6 cells in the presence of 30 μg of each plasmid (Coppola et al., 1999). Immediately after electroporation, the cells were resuspended in culture medium and distributed in 24-multiwell plates at a concentration of approximately 3×10^5 cells/well. The expression level of each Rabphilin construct was assessed by western blotting using a mouse monoclonal antibody directed against the HA epitope tag (12CA5).

Subcellular localization of hTfr and VAMP-2

HIT-T15 cells were transiently transfected with hTfr or, for the experiments shown in Fig. 9, with both hTfr and VAMP-2-Tag (Grote and Kelly, 1996). For the experiments in Fig. 1, the cells cultured for 3 days on glass coverslips were pre-incubated in culture medium for 1 hour at 4°C with hTfr coupled to FITC. For the experiments shown in Fig. 9, the cells were pre-incubated at 4°C for 45 minutes with hTfr coupled to FITC, and with a monoclonal antibody directed against the T antigen tag (KT3). After washing, the cells were exposed for 30 minutes at 4°C to a Cy3-labeled anti-mouse antibody in the constant presence of hTfr-FITC.

At the end of the different pre-incubation procedures the coverslips were washed and shifted at 37°C for the indicated times. The cells were fixed in 4% paraformaldehyde and analyzed by confocal microscopy (model TCS NT, Leica, Laserotechnik, Heidelberg, Germany). Excitation was obtained with an Argon-Krypton laser, with line set at 488 nm for fluorescein excitation and 588 nm for rhodamine excitation. The emitted light was filtered through appropriate filters (BF 530/30 for fluorescein, LP 590 for rhodamine). Single section images of 512×512 pixels were taken with a 40× objective, numerical aperture 1.32.

Measurement of transferrin endocytosis

HIT-T15 cells transiently cotransfected with a plasmid encoding the hTfr receptor and with the Rabphilin constructs were seeded in 24-multiwell plates at a concentration of about 2×10^5 cell/well. Three days after transfection, the cells were incubated in culture medium for 60 minutes at 4°C in the presence of 10 μg/ml hTfr coupled to HRP. After this period, the cells were washed once in culture medium and four times in PBS to eliminate unbound hTfr. They were then either kept at 4°C for another 5 minutes or incubated for 2-5 minutes at 37°C in culture medium. After the incubation, the cells were washed three times at 4°C with PBS or with PBS supplemented with 30 mM glycine at pH 2.4. At the end of this procedure, the cells were lysed at room temperature in 100 μl PBS supplemented with 1% Triton-X 100. The amount of hTfr bound to the surface of the cells (washes with PBS) or internalized (washes with PBS supplemented with glycine) was assessed using a colorimetric assay. For this purpose, an aliquot of the lysate (5-10 μl) was diluted in 50 μl 3,3',5,5'-tetramethylbenzidine and incubated for 15 minutes at room temperature. The reaction was stopped by adding 50 μl stop solution containing 1.5 M H2SO4 and 1 M HCl. HRP activity was estimated by measuring the absorbance at 450 nm.

Assessment of the interaction of Rabphilin mutants with Rab3A

The ability of Rabphilin mutants to bind to Rab3A was tested using a mammalian two-hybrid system (CheckMate™, Promega). For this purpose, the full-length cDNAs of the Rabphilin mutants were subcloned in frame with VP16 in the expression vector pACT,
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whereas the constitutively active Rab3A Q81L mutant was subcloned in frame with GAL4 in the expression vector pBIND. The GAL4 and VP16 fusion proteins were cotransfected in HIT-T15 cells along with a third plasmid (pG5lac) encoding five GAL4 binding sites upstream of the firefly luciferase gene. Three days after transfection, the cells were lysed and the amount of firefly luciferase was quantitated using the Dual-Luciferase™ Reporter Assay System (Promega).

**Interaction of Rabphilin mutants with \( \alpha \)-actinin**

Approximately 20 pmol of bacterially expressed fusion proteins between Glutathion-S-transferase (GST) and the N-terminal fragment (amino acids 1-206) of wild-type or mutated Rabphilin were immobilized on glutathion-agarose beads (Sigma). The affinity columns were incubated for 90 minutes at 4°C with 100 pmol of purified \( \alpha \)-actinin in 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.26% CHAPS and 150 mM NaCl. After five washes in the same buffer the proteins remaining attached to the beads were analyzed by SDS-PAGE. The \( \alpha \)-actinin associated with the affinity columns was detected by immunoblotting using a polyclonal antibody directed against the purified protein.

**Interaction of Rabphilin mutants with Rabaptin-5**

For analysis of the ability of different mutants of Rabphilin to bind to Rabaptin-5, the GST-affinity columns described above were incubated in the presence of \([\text{35}S]\)-labeled Rabaptin-5 (amino acids 385-753) generated by in vitro translation (Promega).

**RESULTS**

The involvement of Rabphilin in the regulation of endocytosis was investigated using cells of the hamster insulin-secreting line HIT-T15 that were transiently cotransfected with plasmids encoding the human transferrin receptor and different Rabphilin mutants. As shown in Fig. 1, if 3 days after transfection the cells were incubated at 4°C in the presence of human transferrin (hTfr) coupled to FITC, the plasma membrane of the cells expressing the receptor was fluorescently labeled (Fig. 1A). The binding of hTfr to untransfected hamster cells was undetectable (not shown). As expected, the labeling observed on transfected cells was abolished by acid washes, indicating that, under these conditions, hTfr was retained on the surface of the cells (Fig. 1B). If after the initial labeling at 4°C the cells were incubated for 5 minutes at 37°C, the fluorescence localized on punctate structures inside the cells. In this case the signal was resistant to acid washes confirming that hTfr was internalized (Fig. 1C). To measure the amount of hTfr bound on the cell surface at 4°C or internalized during the incubation at 37°C, we performed experiments using hTfr coupled to horseradish peroxidase. As shown in Fig. 2, the amount of hTfr bound at 4°C in cells transfected with hTfr receptor was four to five times greater than that in cells transfected with the vector alone. As expected, the peroxidase activity was reduced to background levels after acid washes. By contrast, if the cells were incubated for 5 minutes at 37°C prior to the acid treatment, a fraction of the peroxidase activity was internalized and recovered in the cell extract (Fig. 2).

This approach was used to measure the effect on endocytosis of wild-type Rabphilin and of two point mutants, Rabphilin R60A and Rabphilin V61A. The R60A mutant displays wild-type affinity for Rab3 and is targeted to secretory vesicles but has no stimulatory effect on exocytosis (Joberty et al., 1999).
The V61A mutant cannot bind to Rab3 and is targeted to secretory vesicles less efficiently than wild-type Rabphilin but retains the ability to enhance K⁺-induced exocytosis (Joberty et al., 1999). As shown in Fig. 3, hTfr endocytosis was not affected by the overexpression of wild-type Rabphilin or the R60A mutant. By contrast, in cells transfected with the V61A mutant, the amount of hTfr internalized after 2 or 5 minutes at 37°C was approximately doubled. This indicates that Rabphilin is indeed involved in endocytosis and suggests that this function is negatively modulated by interaction with Rab3.

We attempted to clarify the role of Rabphilin in the endocytotic process by taking advantage of another Rabphilin mutant that cannot interact with Rab3. This mutant was selected using a so-called mammalian two-hybrid system. For this purpose, we generated fusion proteins between the transcriptional activator VP16 and Rabphilin, and between Gal4 and Rab3A. These constructs were transiently cotransfected in HIT-T15 cells together with a plasmid encoding the Gal4 binding sequence upstream of the luciferase gene. As expected, transfection of the VP16/Rabphilin or Gal4/Rab3A proteins alone led to a poor expression of the luciferase gene (Fig. 4). However, co-expression of the fusion protein including wild-type Rabphilin with Gal4/Rab3A resulted in a strong induction of the reporter gene (Fig. 4). By contrast, the fusion protein between VP16 and the V61A mutant caused no induction of the luciferase gene, confirming that this mutant cannot bind efficiently to Rab3 (Joberty et al., 1999). A VP16 fusion protein including a Rabphilin mutant in which leucine 83 was replaced by alanine (L83A) was also inactive, indicating that this mutant cannot interact with Rab3 (Fig. 4).

We then tested the effect of the L83A mutant on endocytosis (Fig. 5). In this series of experiments, the V61A mutant enhanced hTfr internalization by about two-fold. This effect was comparable with that obtained after overexpression of wild-type Rabaptin-5, a known component of the endocytotic machinery (Fig. 5). Surprisingly, however, the L83A mutant had no significant effect on endocytosis.

In an attempt to elucidate the cause(s) of the different behaviors of Rabphilin V61A and Rabphilin L83A on endocytosis, the properties of the two mutants were analyzed in detail. The lack of effect of Rabphilin L83A was not due to an instability of the protein, because all the Rabphilin constructs used in the study were expressed to similar levels (Fig. 6). We have shown previously that both Rabphilin V61A and Rabphilin L83A have stimulatory effects on K⁺-triggered
Fig. 6. Expression level of Rabphilin constructs in HIT-T15 cells. HIT-T15 cells were transiently transfected with an empty vector (control), wild-type Rabphilin, Rabphilin R60A, Rabphilin V61A and Rabphilin L83A. Three days later the cells were homogenized and the proteins analyzed by western blotting using a monoclonal antibody against the HA epitope tag.

exocytosis (Joberty et al., 1999). In addition, we have reported that in PC12 cells both proteins associate with secretory vesicles but their targeting efficiency is reduced (Joberty et al., 1999). To verify that these observations were not restricted to PC12 cells we analyzed by confocal microscopy the subcellular localization of the two mutants in HIT-T15 cells. We found that both the V61A and the L83A mutants colocalize with the same efficiency with secretory granules (not shown).

The N-terminal domain of Rabphilin has been shown to interact with \( \alpha \)-actinin in a Rab3-dependent manner (Kato et al., 1996). Because the reorganization of the actin cytoskeleton could potentially underlie some of the effects of Rabphilin on the endocytotic process, we investigated the impact of the mutations in valine 61 and in leucine 83 on \( \alpha \)-actinin binding. To this end, we prepared GST-affinity columns containing either GST alone or GST fusion proteins including the N-terminal fragment of Rabphilin. As shown in Fig. 7, \( \alpha \)-actinin did not interact with the affinity column containing GST alone. The columns containing the N-terminal fragment of wild-type and mutated Rabphilin retained the same amount of \( \alpha \)-actinin, indicating that valine 61 and leucine 83 are not directly involved in the binding of this regulatory component of the actin cytoskeleton.

We next tested whether the V61A and the L83A mutants could interact with Rabaptin-5. In this case, GST affinity columns analogous to those used for the experiments in Fig. 7 were incubated in the presence of radioactively labeled Rabaptin-5. Wild-type Rabphilin and the V61A mutant were found to interact with similar efficiency with Rabaptin-5 (Fig. 8). By contrast, Rabaptin-5 was not retained on affinity columns containing GST alone or including the L83A mutant (Fig. 8). This indicates that leucine 83 is essential for interaction with both Rab3 and Rabaptin-5.

The recycling of the granule components after insulin exocytosis is thought to occur by a process similar to receptor-mediated endocytosis. This process has, however, not been studied in detail. For this reason, we investigated whether the vesicle membrane protein VAMP-2 is co-internalized with the transferrin receptor. To this end, we co-expressed in HIT-T15 cells the human transferrin receptor and a VAMP-2 construct containing a C-terminal T antigen tag. When the secretory vesicles fuse with the plasma membrane this tag is exposed to the exterior of the cell (Grote and Kelly, 1996). Thanks to this property, it is possible to follow the fate of VAMP-2 after exocytosis with an antibody directed against the tag that is added in the extracellular medium. As shown in Fig. 9, after a 1 minute incubation at 37°C, human transferrin and VAMP-2 were internalized in small punctate structures. Comparison of the two confocal images revealed that part of the endosomal structures positive for transferrin were labeled by the antibody against the tag of VAMP-2. The colocalization of the two proteins indicates that, at least in its initial steps, the recycling of VAMP-2 occurs by a mechanism analogous to that of receptor-mediated endocytosis.

**DISCUSSION**

Exocytosis and endocytosis are two inter-related processes that need to be coordinated to preserve the efficiency of the secretary apparatus. This is most dramatically demonstrated in nerve terminals, where genetic defects that affect endocytosis cause a rapid depletion of the ready releasable pool of synaptic vesicles (Koenig and Ikeda, 1989). However, tight coupling between exo- and endocytosis is likely to be required in all secretary systems. Indeed, recent evidence obtained in chromaffin cells suggests that recycling of the membrane components of dense core secretory granules is required for the biogenesis of secretory vesicles (Slembrouk et al., 1999). During the past few years, several components of the molecular machinery involved in exocytosis and endocytosis have been
identified, but the mechanism insuring the coupling between the two pathways is still unclear. In this study, we provide evidence that Rabphilin, a putative player in the exocytotic process (Chung et al., 1995; Arribas et al., 1997; Joberty et al., 1999), is also involved in endocytosis.

Our findings are consistent with the results obtained in squid giant synapses and PC12 cells. In squid giant synapses, microinjection of full-length Rabphilin increases the membrane area of endosomes (Burns et al., 1998). In PC12 cells, the N-terminal fragment of Rabphilin was reported to inhibit transferrin endocytosis (Ohya et al., 1998). Using a transient transfection system, we found that a mutant of Rabphilin that is unable to form a complex with Rab3 increases transferrin internalization. By contrast, wild-type Rabphilin was inactive. The most likely explanation for this finding is that the function of wild-type Rabphilin is negatively modulated by endogenous Rab3. The loss of this inhibitory action would enable the mutants that cannot bind to the GTPase to stimulate transferrin internalization. The pool of Rab3 associated with secretory vesicles is known to be predominantly in the GTP-bound conformation (Stahl et al., 1994; Burstein et al., 1993). Therefore, under resting conditions, the majority of Rabphilin attached to secretory vesicles is expected to be complexed to Rab3 (Fig. 10). When exocytosis is triggered, the GTPase activity of Rab3 is enhanced leading to the hydrolysis of GTP and the release of inorganic phosphate (Pi) (Stahl et al., 1994). Thus, during or immediately after the fusion of secretory vesicles with the plasma membrane, Rab3 switches from the GTP-bound to the GDP-bound state. The conformational change associated with the hydrolysis of GTP will cause a drop in the affinity for Rabphilin (Shiratani et al., 1993), favoring the formation of a soluble Rab3-RabGDI complex (Matsui et al., 1990). Thus, immediately after the insertion of the secretory vesicle membrane in the plasma membrane, Rabphilin becomes free to interact with other binding partners and to trigger endocytosis (Fig. 10). This scenario provides a possible explanation for the spatio-temporal link between exo- and endocytosis. In this study, the role of Rabphilin in endocytosis was investigated by measuring transferrin internalization. However, because immediately after internalization we observe a partial colocalization of VAMP-2 and transferrin on the same endosomal compartment, Rabphilin will most likely accelerate the recycling of secretory vesicle proteins.

Rabphilin has been shown to bind to Rabaptin-5 (Ohya et al., 1998), a known component of the endocytotic machinery (Stenmark et al., 1995). The association between the two proteins occurs through the N-terminal portion of Rabphilin and is prevented by the binding of Rab3 (Ohya et al., 1998). Our results confirm these findings and narrow down the domain of interaction to a portion of the amphipathic α-helix at the N-terminus of Rabphilin. Taking advantage of the properties of different Rabphilin mutants, we show that the interaction with Rabaptin-5 is required for the stimulation of endocytosis. Rabaptin-5 and the guanine nucleotide exchange factor Rabex-5 are known to form a complex that activates the GTPase Rab5 (Horiuchi et al., 1997). This leads to the assembly of a multiprotein complex including the early endosome-associated protein EEA1 and phosphatidylinositol 3-kinase that promotes the fusion of clathrin-coated vesicles with early endosomes, and the homotypic fusion of early endosomes (McBride et al., 1999; Christoforidis et al., 1999). We propose that immediately after the fusion of secretory vesicles with the plasma membrane, Rabphilin dissociates from Rab3 and binds to Rabaptin-5 (Fig. 9). This would cause the recruitment of an important component of the machinery involved in the budding

Fig. 9. Subcellular localization of hTfr and VAMP-2 after internalization. HIT-T15 cells were transiently transfected with a plasmid encoding the hTfr receptor and with a VAMP-2 construct containing a T antigen tag that is exposed to the luminal side of secretory vesicles (Grote and Kelly, 1996). Three days after transfection the cells were pre-incubated at 4°C with FITC-coupled hTfr and with a monoclonal antibody against the T antigen tag. The monoclonal antibody was then labeled by exposing the cells to a Cy3-coupled anti-mouse antibody. At the end of the pre-incubation the cells were transferred for 1 minute at 37°C, washed with PBS supplemented with 30 mM glycine at pH 2.4 and fixed for analysis at the confocal microscope. The figure shows a single section image.

Fig. 10. Model for the role of Rabphilin in the coupling of exo- and endocytosis. Under resting conditions, Rabphilin is localized on the membrane of secretory vesicles and is tightly associated to the GTP-bound form of Rab3. Upon vesicle fusion, Rab3 hydrolyses GTP and forms a complex with RabGDI that dissociates from the membrane. This permits Rabphilin to recruit Rabaptin-5 near the exocytotic site, thus contributing to the rapid recycling of the components of the secretory vesicle.
of endocytic vesicles close to the release site. Rabaptin-5 together with Rabex-5 could then activate Rab5 and, in turn, promote the assembly of the multiprotein complex controlling the fusion of clathrin-coated vesicles with early endosomes. This hypothesis is in agreement with the fact that overexpression of Rabphilin V61A, Rabaptin-5, or a constitutively active mutant of Rab5 (Stenmark et al., 1994) all have similar effects on transferrin endocytosis.

Rabphilin is not the first example of a protein associated with the membrane of secretory vesicles that is proposed to generate exocytosis. Synaptotagmin was found to be a high-affinity binding site for the clathrin adaptor complex AP-2 (Zhang et al., 1994). Genetic evidence obtained in Caenorhabditis elegans confirmed the involvement of synaptotagmin in synaptic vesicle recycling (Jorgensen et al., 1995). Newly assembled clathrin-coated pits are unable to proceed to ligand sequestration in the absence of Rab5 (McLauchlan et al., 1997). Thus, it is possible that the recycling of secretory vesicles requires the concerted action of both synaptotagmin and Rabphilin. Synaptotagmin would initiate the assembly of the clathrin coat by binding to AP-2, whereas Rabphilin would recruit Rabaptin-5 and its partners.

In summary, we have identified Rabphilin as a possible candidate for coordination of the exo- and endocytic pathways. Future studies will have to elucidate the precise sequence of events that are elicited by Rabphilin in order to increase the internalization rate of clathrin-coated vesicles. This important task will certainly be facilitated now we know more about the characteristics of different point mutants of Rabphilin.

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