VAMP4 cycles from the cell surface to the trans-Golgi network via sorting and recycling endosomes

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Summary

VAMP4 is enriched in the trans-Golgi network (TGN) and functions in traffic from the early and recycling endosomes to the TGN, but its trafficking itinerary is unknown. Cells stably expressing TGN-enriched VAMP4 C-terminally-tagged with EGFP (VAMP4-EGFP) are able to internalize and transport EGFP antibody efficiently to the TGN, suggesting that VAMP4-EGFP cycles between the cell surface and the TGN. The N-terminal extension of VAMP4 endows a chimeric VAMP5 with the ability to cycle from the surface to the TGN. Detailed time-course analysis of EGFP antibody transport to the TGN as well as pharmacological and thermal perturbation experiments suggest that VAMP4-EGFP is endocytosed by clathrin-dependent pathways and is delivered to the sorting and then recycling endosomes. This is followed by a direct transport to the TGN, without going through the late endosome. The di-Leu motif of the TGN-targeting signal is important for internalization, whereas the acidic cluster is crucial for efficient delivery of internalized antibody from the endosome to the TGN. These results suggest that the TGN-targeting signal of VAMP4 mediates the efficient recycling of VAMP4 from the cell surface to the TGN via the sorting and recycling endosomes, thus conferring steady-state enrichment of VAMP4 at the TGN.

Supplementary material available online at
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Key words: VAMP4, SNARE, Endosome, Golgi complex, TGN, Membrane targeting

Introduction

Protein transport in the secretory and endocytic pathways is mediated by membrane-enclosed intermediates in the form of small vesicles or larger containers. These shuttling vehicles are generated from a donor compartment and delivered to the target compartment. After a process referred to as tethering, these intermediates are docked and then fused with the target compartment in a process catalyzed by SNARE (soluble N-ethyl maleimide sensitive factor adaptor protein receptor) (Söllner et al., 1993; McNew et al., 2000; Bonifacino and Glick, 2004; Hong, 2005). SNARE-mediated fusion of vesicles/containers with a target compartment is evolutionally conserved from yeast to human and is used in diverse transports in the secretory and endocytic pathways (Weimbs et al., 1997; Fasshauer et al., 1998; Hong, 2005). For a given transport, v-SNARE associated with the vesicle interacts with t-SNARE on the target compartment to form a trans-SNARE complex between two apposing membranes (Sutton et al., 1998; Weber et al., 1998; Antonin et al., 2002). t-SNARE generally consists of a heavy chain and two light chains, thus providing three SNARE motifs (Fukuda et al., 2000; Hong, 2005). During the formation of a typical trans-SNARE complex, the four SNARE motifs (one from v-SNARE and three from t-SNARE) assemble into a four-helical bundle to catalyze the fusion event (Söllner et al., 1993; Weber et al., 1998; Bock et al., 2001; Chen and Scheller, 2001; Antonin et al., 2002; Jahn et al., 2003).

The function of SNAREs is best defined for exocytosis of synaptic vesicles. Fusion of synaptic vesicles with the presynaptic membrane of neurons is mediated by interaction of VAMP2/synaptobrevin as a v-SNARE with a t-SNARE assembled from syntaxin1 and SNAP-25 (Söllner et al., 1993; Sutton et al., 1998; Jahn et al., 2003; Sudhof, 2004). Syntaxin1 and VAMP2 have one SNARE motif each, whereas SNAP-25 has two tandem SNARE motifs. The structure resolved by x-ray crystallography revealed that the four-helical bundle assembled from the four SNARE motifs is characterized by 16 layers of mostly hydrophobic interactions between amino acid side chains (Sutton et al., 1998; Antonin et al., 2002). The central zero layer is defined by a cluster of hydrophilic interactions between three Gln (Q) residues (derived from t-SNARE) and one Arg (R) residue (derived from v-SNARE). Based on the residue predicted to reside at the zero layer, SNAREs can be classified into either Q-SNAREs or R-SNAREs (Fasshauer et al., 1998). After membrane fusion, the SNARE complex becomes a cis complex in the target compartment and is disassembled into free v-SNARE and t-SNARE subunits via the action of NSF (N-ethylmaleimide-sensitive factor) and α-SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein). The v-SNARE is generally recycled back to the donor compartment, whereas the t-SNARE subunits are reused for the next rounds of fusion (Hohl et al., 1998; Wimmer et al., 2001; Marz et al., 2003).

Most of the nine known R-SNAREs (Sec22b, Ykt6, VAMP1, VAMP2, VAMP3/cellubrevin, VAMP4, VAMP5, VAMP7/Ti-VAMP, VAMP8/endobrevin) function as v-

SNAREs and they are distributed in different compartments of the secretory and endocytic pathways (Chen and Scheller, 2001; Hong, 2005). Sec22b is enriched in the transport intermediates between the ER and the cis-Golgi (Hay et al., 1997; Paek et al., 1997; Zhang et al., 1999), whereas Ykt6 is enriched in the cis-Golgi and Golgi stack (Sögaard et al., 1994; McNew et al., 1997; Zhang and Hong, 2001; Xu et al., 2002). The seven VAMPs are major v-SNAREs operating in post-Golgi compartments with VAMP1 and VAMP2 functioning in regulated exocytosis of neurons and endocrine cells (Jahn and Sudhof, 1999; Lin and Scheller, 2000), whereas VAMP3 is enriched in the sorting and recycling endosomes (McMahon et al., 1993; Galli et al., 1994; Lin and Scheller, 2000). VAMP4 is predominantly localized to the TGN and has also been found in endosomes (Advani et al., 1998; Steegmaier et al., 1999; Mallard et al., 2002). Being enriched on the plasma membrane, VAMP5 is mainly expressed in the skeletal muscle and heart and its expression level is enhanced during in vitro myogenesis of C2C12 cells (Zeng et al., 1998). VAMP7 is enriched in late endosomal compartments and the lysosomes and is also involved in neurite outgrowth (Advani et al., 1998; Martinez-Arca et al., 2000; Martinez-Arca et al., 2001). VAMP8 is enriched in the early and late endosomes, but recent studies indicated that it also functions as a v-SNARE for regulated exocytosis of exocrine tissues such as pancreatic acinar cells (Wong et al., 1998; Antonin et al., 2000; Wang, C. C. et al., 2004). Accordingly, the targeting and trafficking of these R-SNAREs and Q-SNAREs represent an important aspect of the regulation of SNARE function. Newly made t-SNAREs must be delivered to the proper compartments to function, whereas all vesicles derived from donor compartments must be equipped with the right v-SNARE to fuse with the right target compartments. Recycling of v-SNAREs to donor compartments after fusion might be important to ensure tight regulation of its function.

We and others have found that the N-terminal extension of VAMP4 contains an autonomous signal for targeting to the TGN and that this signal consists of a double leucine (or di-Leu) motif followed by an acidic cluster (Peden et al., 2001; Zeng et al., 2003; Hinners et al., 2003). In addition, it was shown that VAMP4 interacts with syntaxin 16, syntaxin 6 and Vti1a to form a SNARE complex that mediates traffic from the sorting and recycling endosomes back to the TGN (Mallard et al., 2002). Despite these understandings, we know little about the cellular itinerary and dynamics of VAMP4. In this report, we present results showing that VAMP4 cycles efficiently from the plasma membrane back to the TGN via the sorting and then recycling endosome, and that its TGN-targeting signal is important for this recycling.

Results

The N-terminus of VAMP4 mediates recycling from the surface to the TGN

To follow the recycling of VAMP4 from the surface to the TGN, NRK cells stably transfected to express VAMP4-EGFP were incubated with culture medium containing rabbit antibody against EGFP at 37°C for 60 minutes. Antibody bound to surface VAMP4-EGFP will travel together with VAMP4-EGFP, thus providing a useful assay for monitoring

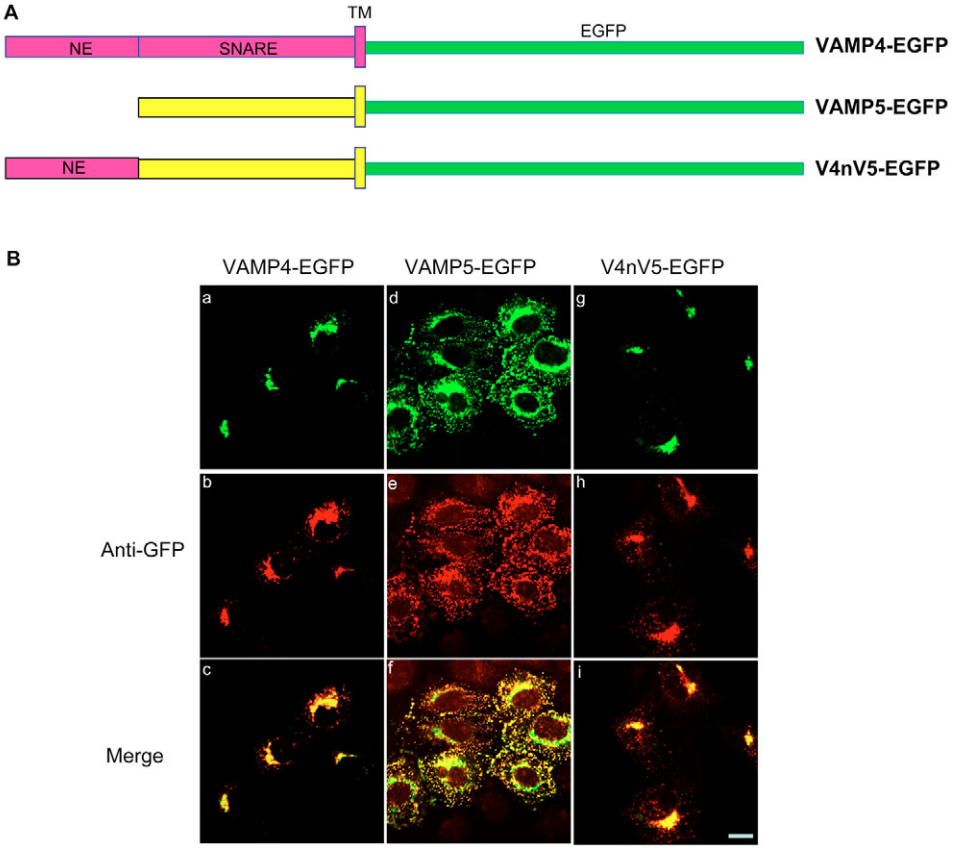

Fig. 1. The N-terminus of VAMP4 mediates recycling from the cell surface to the TGN. (A) EGFP-fusion protein expression constructs. The entire coding region of VAMP4 and VAMP5 was inserted into the pEGFP-N1 vector so that VAMP4 and VAMP5 are expressed as C-terminally EGFP-tagged proteins. The coding region of the N-terminal extension (residues 1-48) of VAMP4 was fused in-frame with the second residue of VAMP5 to create a construct for expressing V4nV5-EGFP (which consists of VAMP4 N-terminal extension fused to VAMP5-EGFP). NE, N-terminal extension; SNARE, SNARE domain; TM, transmembrane domain. NRK cells were then transfected with each of these constructs and pools of stably transfected cells were used in subsequent experiments. (B) NRK cells stably expressing VAMP4-EGFP (a-c), VAMP5-EGFP (d-f) and V4nV5-EGFP (g-i) were incubated at 37°C in the continuous presence of anti-EGFP antibody for 60 minutes. Panels a,d,g show the EGFP signals of these fusion proteins. Anti-EGFP antibody was detected by Cy3-conjugated goat anti-rabbit secondary antibody (b,e,h). The merged images are also shown (c,f,i). Bar, 10 μm.

the recycling from the surface to the TGN. This strategy has been used earlier to follow the traffic of TGN38 from the cell surface to the TGN via sorting and recycling endosomes as well as furin from the cell surface to the TGN via the late endosomes (Ghosh et al., 1998; Mallet and Maxfield, 1999). As reported earlier (Zeng et al., 2003), VAMP4-EGFP exhibited a typical Golgi distribution that was shown to be the TGN (Fig. 1Ba). Furthermore, like endogenous VAMP4 (Mallard et al., 2002), VAMP4-EGFP can interact with endogenous syntaxin 6, syntaxin 16 and Vti1a to form a SNARE complex (supplementary material Fig. S1). Significantly, the majority of internalized antibody colocalized with VAMP4-EGFP in the TGN (Fig. 1Bb). Some antibody molecules were also seen in vesicular structures characteristic of endosomes (Fig. 1Bb,c), suggesting that endosomes could be intermediates in the recycling from the surface to the TGN. This is consistent with the fact that the antibody was internalized continuously and is expected to mark transport intermediates in addition to the site of the steady-state accumulation.

In contrast to the Golgi localization of VAMP4-EGFP, VAMP5-EGFP is predominantly distributed to the cell surface and some vesicular structures in the Golgi and peripheral regions (Fig 1Bd), which is consistent with earlier reports (Zeng et al., 1998; Zeng et al., 2003). Cells expressing VAMP5-EGFP did not concentrate internalized antibody at the Golgi (Fig. 1Be-f), the majority of antibody remained associated with the cell surface as the antibody labeling could be removed by surface stripping with acidic buffer. Consistent with earlier observations (Zeng et al., 2003), V4nV5-EGFP, in which the N-terminal extension of 48 residues of VAMP4 was fused to the N-terminus of VAMP5-EGFP, is predominantly associated with the Golgi complex (Fig. 1Bg). Significantly, cells expressing V4nV5-EGFP can internalize and concentrate antibody in the Golgi complex in addition to the presence of some antibody in the vesicular structures (Fig. 1Bh-i), suggesting that the N-terminal extension of VAMP4 not only confers Golgi targeting of VAMP5-EGFP but also endows upon it the ability to recycle efficiently from the surface to the Golgi complex.

VAMP4-EGFP and V4nV5-EGFP were transported to the Golgi complex via vesicular intermediates

The presence of some antibody in peripheral vesicular structures when cells expressing VAMP4-EGFP and V4nV5-EGFP were allowed to internalize antibody continuously (Fig. 1Bb,h), indicates that these vesicular structures might represent intermediates involved in transport of VAMP4-EGFP and V4nV5-EGFP from the surface to the Golgi. To investigate this possibility, we followed the kinetics and

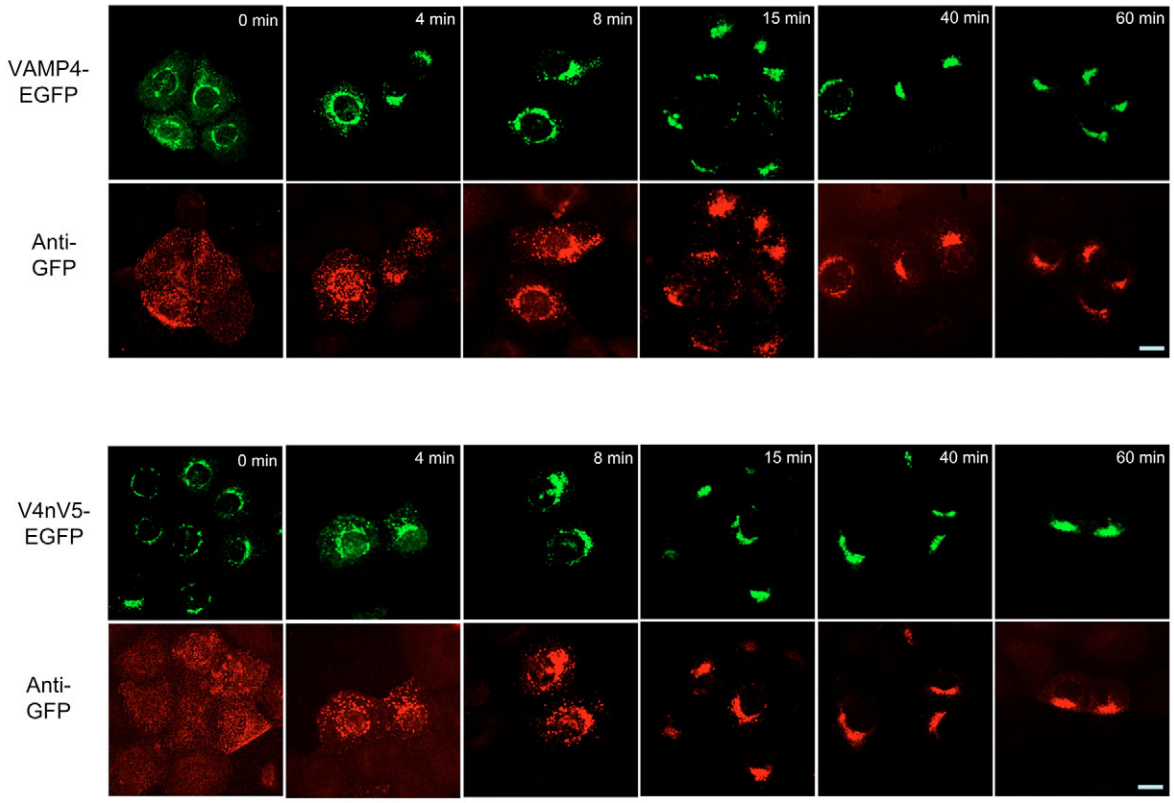

Fig. 2. Involvement of vesicular intermediates in VAMP4-EGFP recycling. NRK cells expressing VAMP4-EGFP (upper panels) or V4nV5-EGFP (lower panels) were incubated with anti-EGFP antibody at 4°C for 1 hour to allow binding to surface fusion proteins. After a brief washing, cells were incubated at 37°C in the absence of antibody for the indicated periods of time to follow the trafficking of fusion protein-bound antibody. EGFP-fusion protein fluoresces green, whereas anti-EGFP antibody was labeled with secondary antibodies conjugated to Cy3 (red). As revealed, recycling of VAMP4-EGFP and V4nV5-EGFP involves peripheral and peri-Golgi vesicular intermediates before being concentrated in the Golgi complex. Bars, 10 μm.

subcellular structures involved in one round of transport of the antibody from the surface to the Golgi. Cells expressing VAMP4-EGFP were allowed to bind anti-EGFP antibody on ice and unbound antibody was washed off. The fate of the surface-bound antibody was then examined after increasing time periods of incubation at 37°C. The antibody was first detected in fine dotted structures distributed throughout the entire cell surface before the culture was warmed up to 37°C (Fig. 2, upper panels, 0 minute), whereas the majority of VAMP4-EGFP was mainly confined to the Golgi. After incubation at 37°C for 4 minutes, antibody was detected in larger vesicular structures in both peri-Golgi and peripheral regions. Antibody labeling became more and more enriched in the Golgi after 8-15 minutes of incubation at 37°C with concomitant decrease of antibody labeling in the peripheral vesicular structures. The majority of the antibody was found concentrated in the Golgi complex from 30 minutes onward. These results suggest that recycling of VAMP4-EGFP involves internalization from the cell surface followed by vesicular intermediates that move from the peripheral to the peri-Golgi region and eventually to the Golgi complex. Similar results were observed when cells expressing V4nV5-EGFP were examined (lower panels, Fig. 2), suggesting that the N-terminal extension can mediate endocytosis from the surface followed by its transport to the Golgi complex via vesicular intermediates.

Clathrin-mediated endocytosis of VAMP4-EGFP

To delineate the molecular aspects of VAMP4-EGFP recycling, we first investigated the mode of VAMP4-EGFP endocytosis from the surface. General inhibitors of clathrin-mediated endocytosis were used to see whether VAMP4-EGFP was internalized by this pathway. Potassium depletion and hypertonic treatment, two known inhibitors of clathrin-mediated endocytosis (Larkin et al., 1983; Daukas and Zigmond, 1985; Madshus et al., 1987; Heuser and Anderson, 1989; Hansen et al., 1993), were used (Fig. 3). When cells depleted of potassium were allowed to internalize antibody in potassium-free medium, the antibody remained in fine spotty structures distributed throughout the cell surface after 30 minutes of incubation at 37°C (Fig. 3Ab), whereas the majority of VAMP4-EGFP was at the Golgi under this condition (Fig. 3Aa). When cells depleted of potassium were allowed to internalize antibody in K^+-containing medium for the same

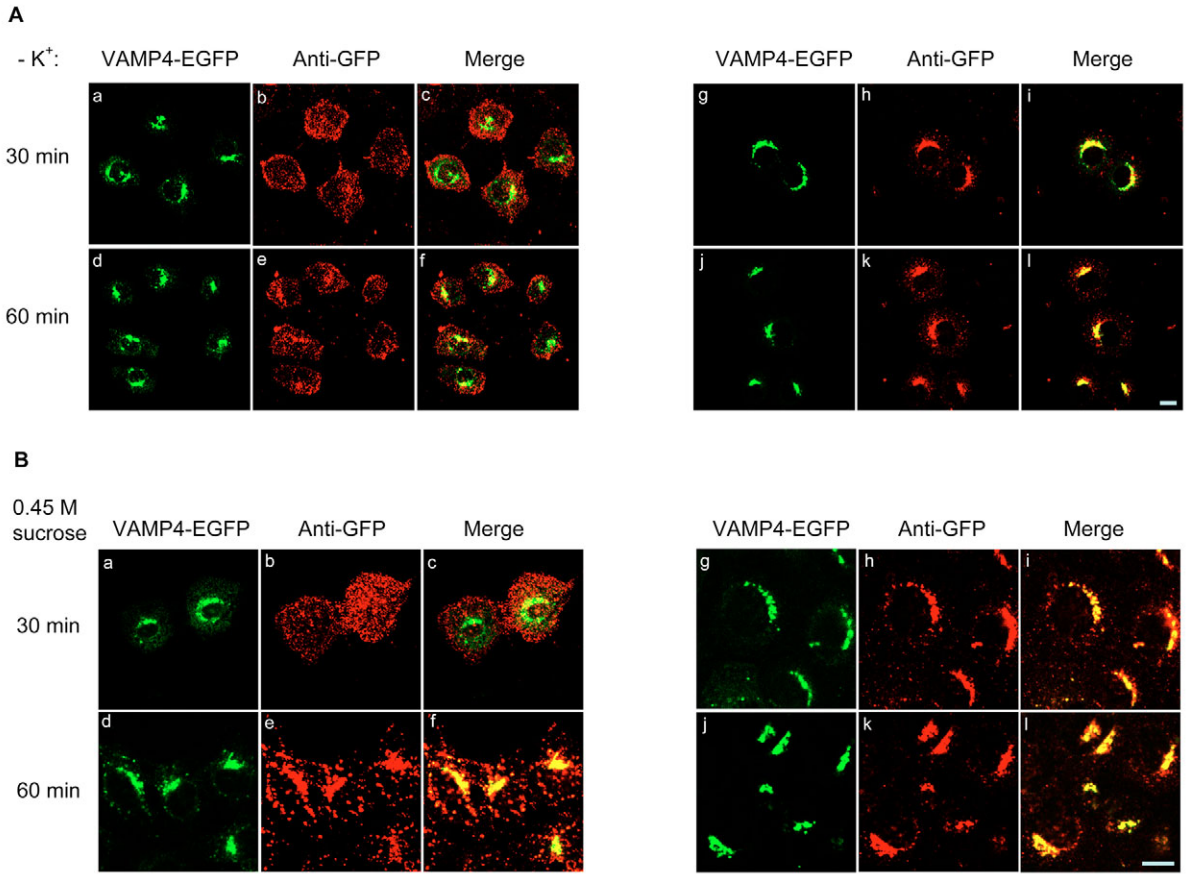

Fig. 3. Inhibition of internalization of VAMP4-EGFP by treatments blocking clathrin-mediated endocytosis. (A) After a hypotonic shock, NRK cells expressing VAMP4-EGFP were incubated with anti-EGFP in the absence (a-f) or presence (g-l) of K^+ for 30 (a-c and g-i) or 60 minutes (d-f and j-l). EGFP-fusion protein fluoresces in green, while anti-EGFP antibody was labeled with secondary antibodies conjugated to Cy3 (red). The merged images are also shown (c,f,i and l). (B) After hypertonic treatment, NRK cells expressing VAMP4-EGFP were incubated with anti-EGFP in the presence (a-f) or absence (g-l) of 0.45 M sucrose for 30 (a-c and g-i) or 60 minutes (d-f and j-l). EGFP-fusion protein fluoresces in green and anti-EGFP antibody was labeled with secondary antibodies conjugated to Cy3 (red). The merged images are also shown (c,f,i,l). Bars, 10 μm.

period of time, efficient delivery of antibody to the Golgi was observed (Fig. 3Ah). The majority of the antibody remained distributed at the surface, even after a 60-minute incubation in K^+-depleted medium (Fig. 3Ae). Similarly, the antibody was detected primarily on the surface when cells grown in hypotonic medium were allowed to internalize antibody for 30 minutes in hypertonic medium (Fig. 3Bb) (see Materials and Methods). By contrast, cells grown in hypertonic medium transported surface-bound antibody to the Golgi complex when incubated in normal medium (Fig. 3Bh). Even after 60 minutes of incubation, the majority of antibody failed to be delivered to the Golgi in hypertonic buffer (Fig. 3Be) and the antibody was instead distributed in peripheral large vesicles. These results suggest that VAMP4-EGFP is internalized primarily via clathrin-mediated endocytosis, which is inhibited in K^+-depleted and hypertonic conditions. Consistent with this conclusion, endocytosis of anti-EGFP antibody was inhibited by a dominant negative mutant (EH29) of EPS15 (Benmerah et al., 1998; Benmerah et al., 1999; Benmerah et al., 2000) (supplementary material Fig. S2) or when clathrin heavy chain was knocked down by small interfering RNA (supplementary material Fig. S3).

VAMP4-EGFP was transported to the Golgi via sorting and recycling endosomes

We then examined the nature of vesicular intermediates involved in transport of VAMP4-EGFP to the Golgi. Internalized antibody was double-labeled with markers of

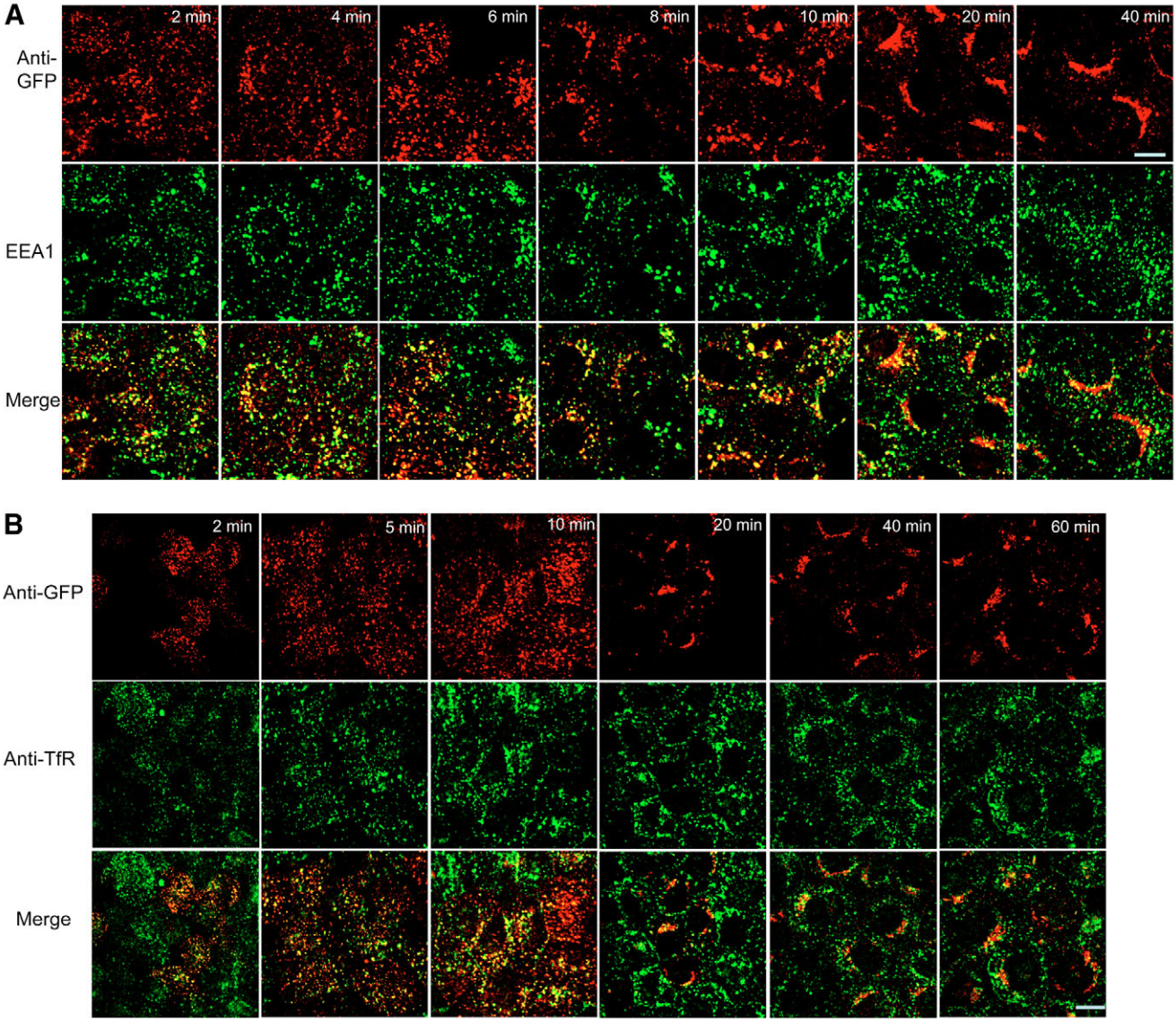

Fig. 4. VAMP4-EGFP recycles through the sorting and recycling endosomes labeled by EEA1 and transferrin receptor (TfR). (A) NRK cells expressing VAMP4-EGFP were incubated with anti-EGFP antibody at 4°C for 1 hour to label the surface pool of VAMP4-EGFP. Cells were then incubated at 37°C in the absence of antibody for 2-40 minutes. Endogenous EEA1 is shown in green, while anti-EGFP antibody is shown in red. The merged images are also shown. (B) NRK cells expressing VAMP4-EGFP were incubated with anti-EGFP antibody and anti-TfR at 4°C for 1 hour. After washing, cells were then incubated at 37°C in the absence of the antibody for 2-60 minutes. Anti-TfR is green and anti-EGFP antibody is red. The merged images are also shown. Bars, 10 μm.

endocytic pathway during the course of its transport to the Golgi. We first examined the distribution of internalized antibody in the sorting endosome marked by endogenous EEA1 (Fig. 4A). Internalized antibody was clearly detected in vesicular intermediates that are positive for EEA1 after internalization of surface-bound antibody for 2-4 minutes (2 and 4 minutes panels). The antibody was mainly distributed in the peripheral vesicular structures during 2-6 minutes of internalization, after which, it moved into more peri-Golgi vesicular intermediates and remained colocalized with EEA1. During the 4- to 10-minute period of internalization, the majority of internalized antibody was seen in EEA1-marked sorting endosomes. The antibody began to segregate away from EEA1-marked sorting endosomes after 15-20 minutes. By 30-40 minutes, the majority of antibody was segregated away from EEA1-postive sorting endosomes and became enriched in the Golgi. These results suggest that endocytosed VAMP4-EGFP was first delivered to the peripheral EEA1-marked sorting endosomes and then to the peri-Golgi intermediates before being transported the Golgi complex.

We also compared simultaneously the distribution of antibody against EGFP (to mark the pathway of VAMP4-EGFP) as well as monoclonal antibody against transferrin receptor (TfR; which is endocytosed by clathrin-dependent pathway followed by transport from the sorting to the recycling endosomes, REs) by allowing cells to internalize both antibodies (Fig. 4B). During the first 5 minutes of incubation, EGFP antibody colocalized well with TfR antibody primarily in peripheral intermediates. After 10 minutes of internalization, a significant amount of EGFP antibody was seen in TfR-antibody-positive peri-Golgi vesicles that are characteristic of the REs and this colocalization at the peri-Golgi region persisted during the 10- to 15-minute period and they became gradually segregated during the 20- to 40-minute period. These results suggest the peri-Golgi intermediates involved in VAMP4-EGFE transport are likely to be the REs. We also performed double-labeling of internalized antibody with LBPA to mark the late endosomes (not shown) and it was noticed that the majority of internalized antibody was transported to the Golgi without transit through the late endosomes, indicating that internalized VAMP4-EGFP is probably transported to the

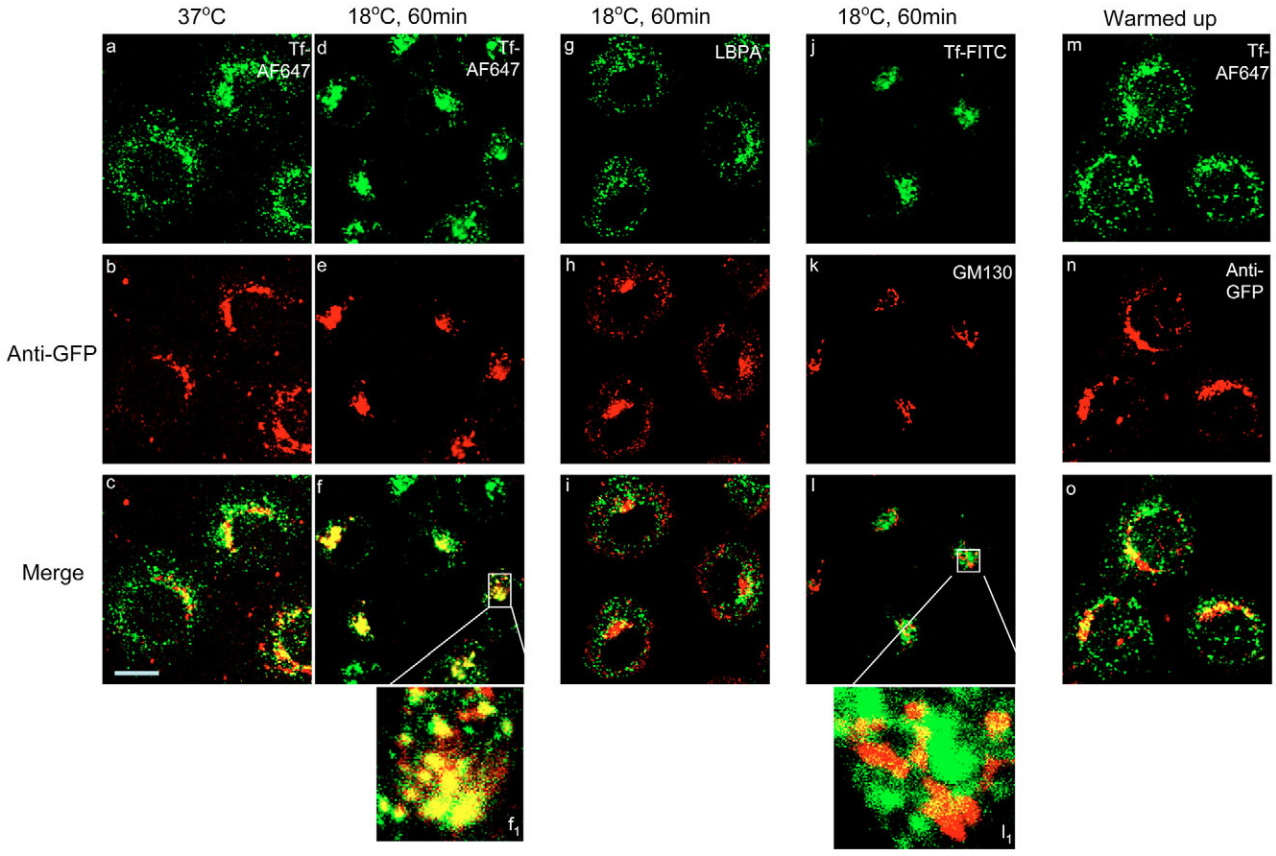

Fig. 5. VAMP4-EGFP recycling was arrested at the peri-Golgi recycling endosomes (REs) at 18°C. Cells expressing VAMP4-EGFP were incubated at 37°C (a-c) or at 18°C (d-i) with either anti-EGFP alone (g-i) or both anti-EGFP and Alexa Fluor 647-conjugated transferrin (Tf-AF647) (a-f) for 1 hour. Anti-EGFP is red (b,e,h) while Tf-AF647 (a,d) or endogenous LBPA (revealed by antibody labeling) (g) is green. The merged images are also shown (c,f,i). Panel f_1 shows the enlarged image of a part of panel f. As a control, NRK cells were incubated at 18°C with FITC-conjugated transferrin (Tf-FITC) for 1 hour (j-l). Cells were then analyzed to view the internalized transferrin (j) and endogenous GM130 as revealed by antibody labeling (k). The merged image is shown in panel l. Panel l_1 shows the enlarged image of a part of panel l, indicating that the peri-Golgi RE are segregated from the Golgi complex. Panels m-o show another control experiment where cells expressing VAMP4-EGFP were incubated at 18°C with Tf-AF647 (green) and anti-EGFP (red) for 1 hour. Cells were then incubated in DMEM at 37°C for an additional 30 minutes before being fixed for viewing. Bar, 10 μm.

Golgi via peripheral sorting endosomes and then peri-Golgi RE.

Further evidence for the involvement of peri-Golgi REs

To verify the possibility that peri-Golgi REs are involved in the transport of VAMP4-EGFP from peripheral sorting endosomes to the Golgi complex, we reasoned that internalized VAMP4-EGFP should be seen to accumulate in the REs if transport from REs to the Golgi is blocked. It has been well established that reduced temperature (such as 18°C) blocked traffic from the REs to the Golgi complex (Mallard et al., 1998; Tai et al., 2004). Accordingly, cells expressing VAMP4-EGFP were allowed to internalize the EGFP antibody simultaneously with Alexa Fluor 647-conjugated transferrin (Tf-AF647) to label the REs at 18°C for 60 minutes (Fig. 5). Internalized antibody was seen to accumulate in peri-Golgi compact structures (Fig. 5e) at 18°C and these structures also accumulate Tf-AF647 (Fig. 5d,f and f_1), suggesting that both internalized antibody and Tf-AF647 were trapped in the peri-Golgi REs. In marked contrast, when cells were allowed to internalize antibody Tf-AF647 at 37°C, the majority of antibody was delivered to the Golgi (Fig. 5b) whereas Tf-AF647 was dynamically distributed in the peri-Golgi RE as well as peripheral endosomes (Fig. 5a). Consistent with the notion that the late endosomes were not involved in traffic of VAMP4-EGFP to the Golgi, VAMP4-EGFP accumulated at peri-Golgi RE (Fig. 5h) did not show any significant overlap with late endosomes marked by LBPA (Fig. 5g). The peri-Golgi REs (shown for Tf in Fig. 5j because both antibody and Tf were almost completely overlapped as shown in Fig. 5f and f_1) was clearly segregated away from the Golgi complex marked by GM130 (Fig. 5k and l_1), further suggesting that transport of internalized antibody from peri-Golgi REs to the Golgi is blocked at 18°C. When cells incubated at 18°C were warmed to 37°C for an additional 30 minutes, antibody and Tf were segregated in that the antibody was transported to the Golgi (Fig. 5n), whereas Tf was re-distributed dynamically in the peri-Golgi REs and peripheral endosomes (Fig. 5m). These results support the notion that peri-Golgi REs is involved in transport of internalized antibody from the sorting endosome to the Golgi complex. Supporting this conclusion was the observation that in cells expressing both VAMP4-EGFP and Tac-TGN38, anti-EGFP antibody was seen to be endocytosed and recycled to the Golgi together with anti-Tac antibody (supplementary material Fig. S4).

Disruption of microtubular network by nocodazole does not affect traffic of Vamp4-EGFP

It has been shown that the disruption of microtubules by nocodazole inhibits the traffic route involving the late endosome, such as transport of furin from the cell surface to the Golgi via the late endosome, but has no significant effect on traffic involving the sorting endosome and the REs (such as traffic of TGN38) (Mallet and Maxfield, 1999). Consistent with the notion that VAMP4-EGFP is transported from the surface to the Golgi via the sorting endosome and then the REs, cells pre-treated with nocodazole were able to internalize antibody efficiently to the fragmented Golgi (Fig. 6b) that was labeled with VAMP4-EGFP (panel a) as well as GS15 (panel d), a Golgi SNARE (Xu et al., 1997; Xu et al., 2002). This observation lends further evidence to the notion that the majority of VAMP4-EGFP is transported from the surface to the Golgi without the involvement of the late endosome.

BFLA1 and concanamycin A inhibit recycling of VAMP4-EGFP at the level of peri-Golgi REs

BFLA1 and concanamycin A (conA) are two known inhibitors of endosomal acidification by inhibiting vacuolar proton pumps (V-ATPase) (Bowman et al., 1988; Dröse et al., 1993). To evaluate the role of endosomal acidification in the traffic of VAMP4-EGFP, we examined the effect of these two inhibitors (Fig. 7). Interestingly, when cells were treated with BFLA1 or conA, both internalized antibody (Fig. 7Ae,h) and AF647-Tf (Fig. 7Ad,g) accumulated in peri-Golgi REs, as they were segregated from the Golgi complex labeled by GM130 (Fig. 7Ak,n,l,o). When the inhibitors were washed off, the antibody was delivered to the Golgi (Fig. 7Bb,e) whereas AF647-Tf was redistributed dynamically between peri-Golgi REs and peripheral endosomes (Fig. 7Ba,b). These observations not only support the conclusion that VAMP4-EGFP is recycled from the surface to the Golgi complex by the sorting endosome and then peri-Golgi REs but also suggest that endosomal acidification inhibited by BFLA1 and conA is essential for traffic from the peri-Golgi REs to the Golgi, highlighting a specific role of V-ATPase in traffic from the REs to the Golgi complex.

Role of TGN-targeting signal in traffic of VAMP4-EGFP

We and others have previously defined a targeting signal in the N-terminal extension of VAMP4 that is necessary and

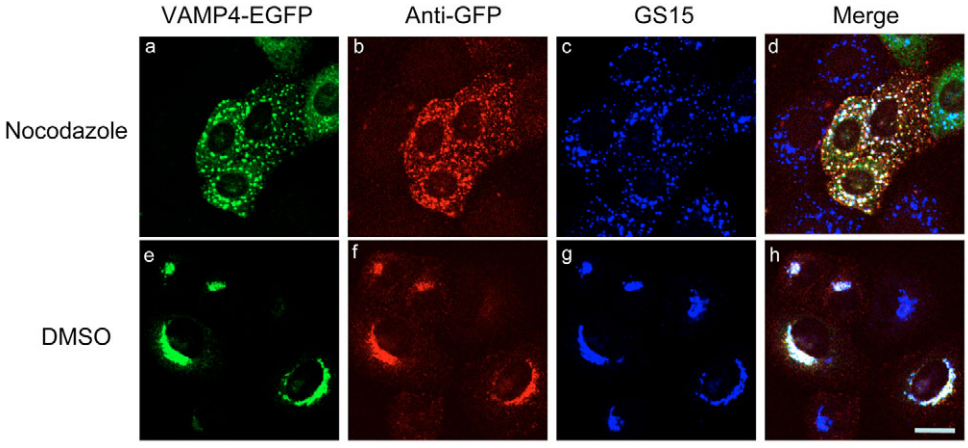

Fig. 6. Nocodazole does not affect VAMP4-EGFP recycling. After a 30-minute pretreatment with 33 μM nocodazole (a-d) or 0.1% DMSO (e-h), VAMP4-EGFP-expressing cells were incubated with rabbit anti-EGFP antibody for 30 minutes at 37°C in the continuous presence of nocodazole (a-d) or DMSO (e-h). Cells were then processed to reveal EGFP (green), anti-EGFP signals (red) and endogenous GS15 (blue). The merged images are also shown. Bar, 10 μm.

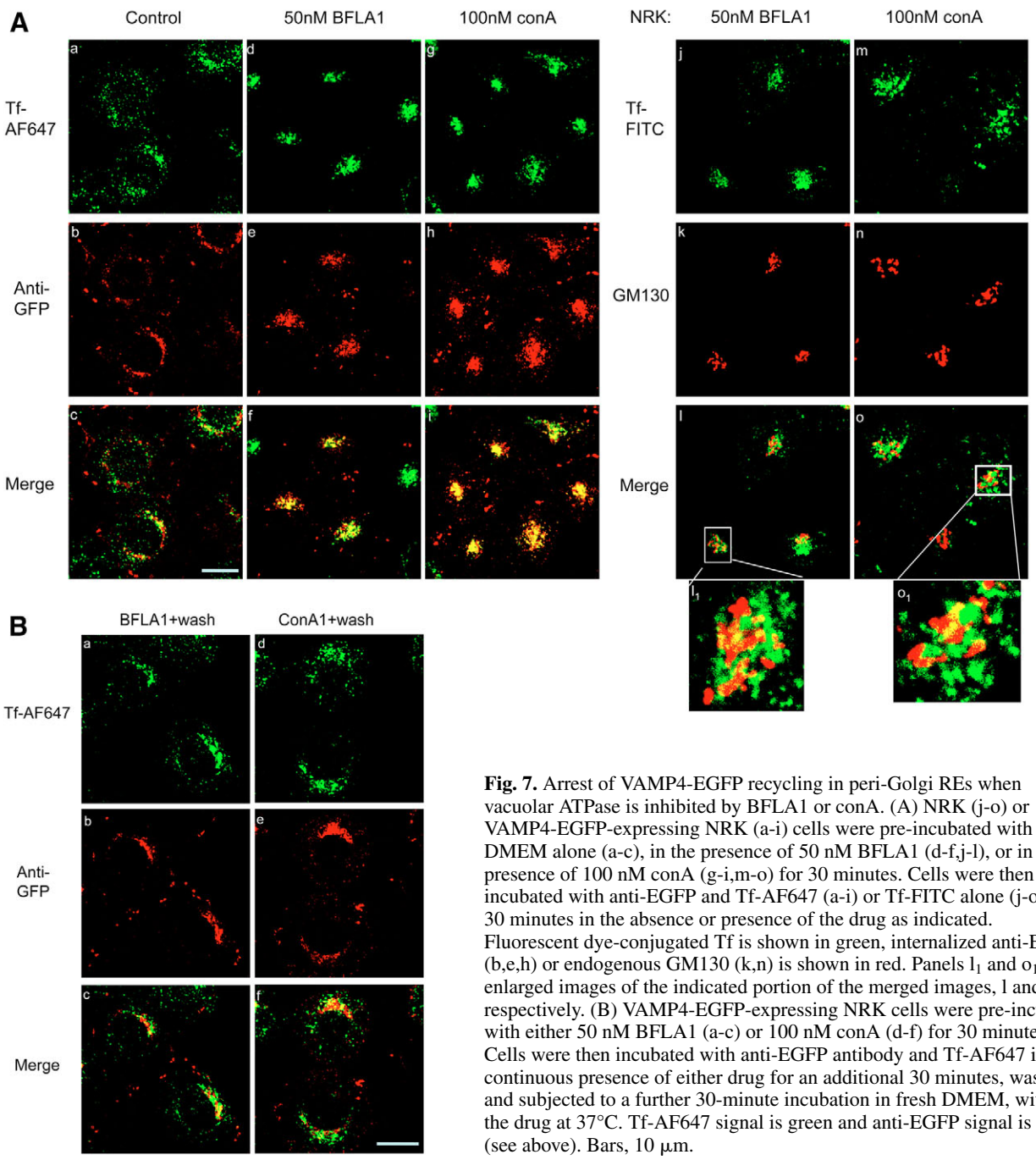

Fig. 7. Arrest of VAMP4-EGFP recycling in peri-Golgi REs when vacuolar ATPase is inhibited by BFLA1 or conA. (A) NRK (j-o) or VAMP4-EGFP-expressing NRK (a-i) cells were pre-incubated with DMEM alone (a-c), in the presence of 50 nM BFLA1 (d-f,j-l), or in the presence of 100 nM conA (g-i,m-o) for 30 minutes. Cells were then incubated with anti-EGFP and Tf-AF647 (a-i) or Tf-FITC alone (j-o) for 30 minutes in the absence or presence of the drug as indicated. Fluorescent dye-conjugated Tf is shown in green, internalized anti-EGFP (b,e,h) or endogenous GM130 (k,n) is shown in red. Panels l_1 and o_1 are enlarged images of the indicated portion of the merged images, l and o, respectively. (B) VAMP4-EGFP-expressing NRK cells were pre-incubated with either 50 nM BFLA1 (a-c) or 100 nM conA (d-f) for 30 minutes. Cells were then incubated with anti-EGFP antibody and Tf-AF647 in the continuous presence of either drug for an additional 30 minutes, washed and subjected to a further 30-minute incubation in fresh DMEM, without the drug at 37°C. Tf-AF647 signal is green and anti-EGFP signal is red (see above). Bars, 10 μm.

sufficient for TGN targeting (Peden et al., 2001; Zeng et al., 2003; Hinners et al., 2003). The signal consists of a di-Leu motif followed by a distal acidic cluster. To evaluate whether the recycling pathway defined here plays a role in governing the steady state distribution of VAMP4 in the TGN, we examined the role of this TGN-targeting signal in the recycling from the surface to the TGN. If TGN targeting of VAMP4 is an event independent of recycling, then this signal should not be crucial for recycling. However, if this recycling directly contributes to the steady-state TGN accumulation of VAMP4, then mutations of this TGN targeting signal are expected to alter the recycling of VAMP4-EGFP from the surface to the TGN. Accordingly, various site-directed mutants of VAMP4 in the context of VAMP4-EGFP (Fig. 8A) were expressed in stably transfected NRK cells and their recycling was examined by antibody uptake and transport (Fig. 8B). These various mutants can be expressed at comparable levels in pooled transfectants when GFP-positive cells were sorted and analyzed (supplementary material Fig. S5). Mutation of the proximal acidic cluster (V4-EDD) or the di-Phe residues (V4-FF) had no major effect on endocytosis and delivery of antibody to the Golgi complex (panels h and n, respectively). However, mutation of the di-Leu motif (V4-LL) completely abolished delivery of antibody to the Golgi complex and antibody was seen in fine dotted structures distributed throughout the entire cells characteristic of surface-bound

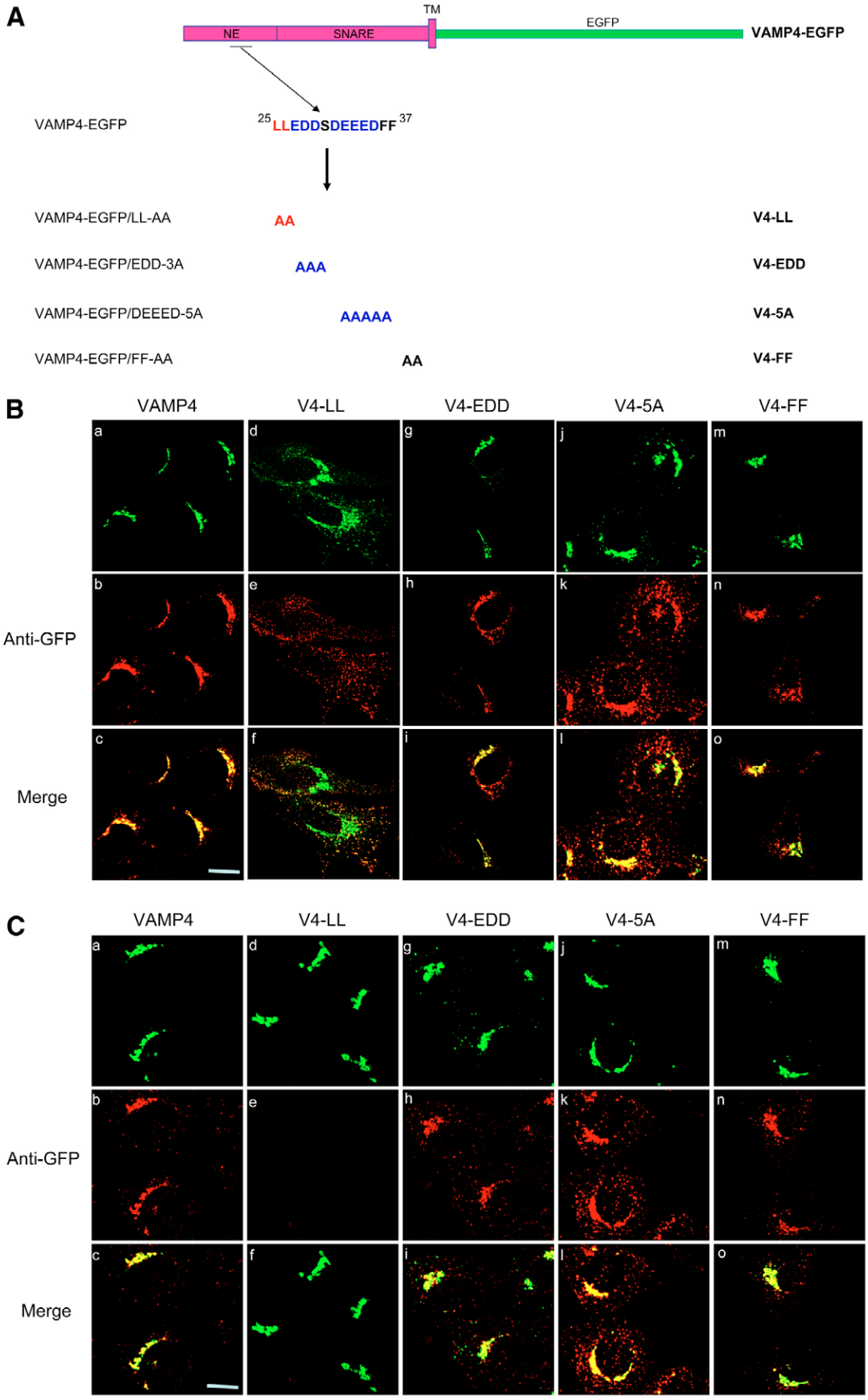

Fig. 8. See next page for legend.

antibody (Fig. 8Be). Consistent with this observation, no antibody labeling was detected in cells expressing V4-LL when surface-bound antibody was removed by acid-stripping after the antibody uptake and delivery assay (Fig. 8Ce). Furthermore, higher surface levels were detected in V4-LL cells compared with V4 cells as assessed by flow cytometry (supplementary material Fig. S6A). These results suggest that the di-Leu motif is essential for internalization of VAMP4-EGFP. Although antibody was still delivered to the Golgi complex in cells expressing mutant that altered the distal acidic cluster (V4-5A) (panel k, Fig. 8B,C), more antibody was clearly found in the vesicular intermediates, indicating either an arrest or delay of VAMP4-EGFP traffic from the sorting endosome and the REs to the Golgi complex. This result indicates that the distal acidic cluster contributes to efficient traffic from endosomal intermediates to the Golgi complex. The strong correlation between the importance of the motifs in TGN targeting shown previously (Zeng et al., 2003), as well as recycling from the surface to the TGN via endosomal intermediates shown here, suggests that this recycling pathway of VAMP4 is important to maintain steady-state enrichment of VAMP4 in the TGN. Furthermore, our results indicate that the di-Leu motif and the distal acidic cluster contribute to TGN targeting by acting at different steps (di-Leu motif in endocytosis while the distal acidic cluster in efficiency of delivery from the vesicular intermediates to the Golgi complex).

Discussion

The following major conclusions can be drawn from our current study. Firstly, VAMP4 is efficiently recycled from the surface to the Golgi complex, which is mediated by clathrin-mediated endocytosis followed by transport to the sorting endosome and then the REs before being concentrated in the Golgi complex. Further support to this trafficking pathway comes from the observation that in CHO cells expressing TacTGN38 and VAMP4-EGFP, mouse monoclonal anti-Tac and rabbit anti-GFP were seen to be co-transported from the surface to the TGN (supplementary material Fig. S4). Additional support for a role of clathrin in mediating endocytosis of VAMP4-EGFP from the surface is the observation that a dominant negative mutant (EH29) of EPS15 (Benmerah et al., 1998; Benmerah et al., 1999; Benmerah et al., 2000) potently blocked endocytosis of surface-bound anti-GFP antibodies (supplementary material Fig. S2) and knockdown of clathrin heavy chain also reduced endocytosis (supplementary material Fig. S3). This recycling pathway is consistent with a defined role of VAMP4 in regulating traffic from the sorting and recycling endosomes to the TGN (Steegmaier et al., 1999; Mallard et al., 2002). Second, a strong correlation of residues important for recycling and steady-state TGN distribution of VAMP4 indicates that this recycling pathway is part of the mechanism governing steady-state enrichment of VAMP4 in the TGN. Accordingly, proteins recognizing these targeting motifs will be part of the machinery involved in this recycling and endosome to Golgi transport. AP-1, PACS-1 and the 39 kDa subunit (physophilin or p39) of V-ATPase have all been shown to interact with VAMP4 (Steegmaier et al., 1999; Peden et al., 2001; Hinners et al., 2003) and they are candidate proteins involved in the trafficking of VAMP4. Thirdly, acidification mediated by V-ATPase is not important for endocytosis and delivery to the endosomes but is specifically required for transport of VAMP4 from the REs to the Golgi, because treatment with V-ATPase inhibitors resulted in an accumulation of internalized antibody in the REs in much the same way as the reduced temperature at 18°C, suggesting a specific role of V-ATPase in RE-TGN traffic.

Immunoelectron microscopy studies have previously shown that VAMP4 is concentrated predominately at the TGN where it is present in clathrin-coated vesicles. However, no detectable labeling of VAMP4 is apparent at the cell surface (Steegmaier et al., 1999). We believe that these results are not inconsistent with our results because the low and transient appearance of recycling VAMP4 on the surface may limit its detection by immunogold labeling. Furthermore, the levels of endogenous VAMP4 in many cells are not high enough, resulting in the detection of VAMP4 mainly in the TGN where it is accumulated. Even in NRK cells stably expressing VAMP4-EGFP, conventional detection of GFP signal did not reveal significant expression of VAMP4-EGFP on the surface unless more sensitive antibody-based flow cytometry was used (supplementary material Fig. S6) or when its endocytosis was inhibited by expression of a dominant-negative mutant of EPS15 (supplementary material Fig. S2). Consistent with this possibility, low and detectable amounts of VAMP4-EGFP and V4nV5-EGFP are accessible to selective surface biotinylation (supplementary material Fig. S7). Previous biochemical studies have shown that VAMP4 can directly interact with AP-1 via its di-Leu motif (Hinners et al., 2003; Peden et al., 2001). Since AP-1 is enriched in the TGN and endosome, AP-1 is unlikely to be involved in the di-Leu motif-mediated endocytosis from the surface. Rather, our results indicate that the di-Leu motif may bridge with clathrin, because endocytosis

Fig. 8. The TGN targeting signal of VAMP4 plays a role in VAMP4-EGFP recycling. (A) The schematic illustration of expression constructs of VAMP4-EGFP and its mutants. Site-directed mutations were created in the context of VAMP4-EGFP. VAMP4-EGFP/LL-AA has the di-Leu motif (residues 25-26) replaced by two Ala residues. The first acidic cluster EDD (residues 27-29) was mutated into three Ala residues in VAMP4-EGFP/EDD-3A. VAMP4-EGFP/DEEED-5A has the distal acidic cluster (DEEED, residues 31–35) replaced by a stretch of five Ala residues. The two Phe residues at positions 36-37 were replaced by Ala residues in VAMP4-EGFP/FF-AA. NE, N-terminal extension; SNARE, SNARE domain; TM, transmembrane domain. These constructs were each transfected into NRK cells and pools of stable transfectants were selected and expanded for the experiments. (B) NRK cells expressing VAMP4-EGFP (a-c), VAMP4-EGFP/LL-AA (panels d-f), VAMP4-EGFP/EDD-3A (panels g-i), VAMP4-EGFP/DEEED-5A (panels j-l) and VAMP4-EGFP/FF-AA (panels m-o) were incubated at 4°C with rabbit anti-EGFP antibody in cold DMEM for 1 hour. After a brief wash, cells were then incubated in fresh DMEM at 37°C for 15 minutes. Panels a,d,g,j,m show the EGFP signal of these fusion proteins. Anti-EGFP antibody was detected by Cy3-conjugated goat anti-rabbit secondary antibody (b,e,h,k,n). The merged images are also shown (c,f,i,l,o). (C) Cells from B were washed twice in cold acidic washing buffer to strip off antibody remaining bound on the surface before being fixed for immunofluorescence microscopy. Panels a, d, g, j and m show the EGFP signal of these fusion proteins. Anti-EGFP antibody was detected by Cy3-conjugated goat anti-rabbit secondary antibody (panels b,e,h,k,n). The merged images are also shown (panels c,f,i,l,o). Bars, 10 μm.

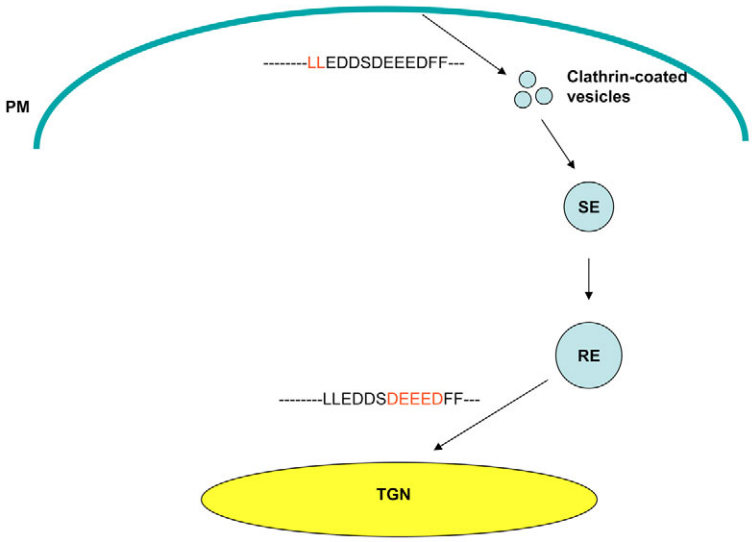

Fig. 9. A schematic model depicting the recycling pathway of VAMP4. VAMP4-EGFP internalized from the surface by clathrin-mediated endocytosis is firstly delivered to the sorting endosomes (SE) labeled with EEA1 and TfR and then transported to the peri-Golgi REs labeled with TfR and revealed by its accumulation there when cells were incubated at 18°C or treated with BFLA1 or conA before being delivered to the TGN. The di-Leu motif of its TGN-targeting signal is necessary for the internalization from the surface whereas the distal acidic cluster is involved in efficient delivery from the endosome to the TGN. The strong correlation of the signal responsible for TGN targeting and recycling indicates that this recycling pathway contributes to the steady-state enrichment of VAMP4 in the TGN. The elucidation of this recycling pathway is in line with the function of VAMP4 as a v-SNARE participating in trafficking from the REs to the TGN.

VAMP4-EGFP is clearly dependent on clathrin. This raises the question as to what is the biological significance of the interaction of the di-Leu motif with AP-1. One possibility is that, other than a role in endocytosis, the di-Leu motif might also play an additional role in the endosome and/or TGN to facilitate the steady-state accumulation of VAMP4 in the TGN. Future study is needed to resolve the role of AP-1 in VAMP4 trafficking.

An earlier study using MDCK cells reported that VAMP4 does not recycle from the surface to the TGN using a similar approach (Steegmeier et al., 2000). The basis for this discrepancy between MDCK and NRK cells is not known. Our earlier studies indicate that intracellular trafficking of SNAREs using exogenous expression in transfected cells is only reliably revealed if we use stably transfected cells, because overexpression of SNAREs in transiently transfected cells often leads to aberrant distribution of SNAREs (Lowe et al., 1997; Subramaniam et al., 2000). Therefore, we have examined the recycling of VAMP4-EGFP and its mutant derivatives in stably transfected NRK, HeLa or CHO cells. Stably transfected cells also have a higher signal/noise ratio as more cells expressing comparable levels of the protein can be studied at the same time. To have more cells that are expressing and to avoid clonal variations, we have used pooled transfectants that were sorted for EGFP-positive cells so that a more homogenous population derived from pooled transfectants were actually used. Accordingly, using stably transfected MDCK cells expressing VAMP4-EGFP, we can similarly detect its recycling using this antibody-uptake approach (not shown).

The observation that the V-ATPase inhibitors BFLA1 and conA arrested transport from the REs to the Golgi complex at the level of peri-Golgi REs in a reversible manner is of interest because it not only highlights a specific role of V-ATPase in the REs but also offers a novel approach to pharmacologically perturb this pathway. This traffic pathway was firstly demonstrated for a TGN protein called TGN38 (Ghosh et al., 1998). The steady-state accumulation of TGN38 in the TGN depends on this recycling pathway, which is also used by several toxins including shiga toxin (via its B subunit), cholera toxin (also via its B fragment) and ricin (Mallard et al., 1998; Sandvig and van Deurs, 2000; Sandvig and van Deurs, 2002) for transport from the outside of the cell to the Golgi complex. These toxins could be retrogradely transported from the Golgi complex to the endoplasmic reticulum (ER), where they are translocated to the cytosol to execute their toxic effects. Recently, several other proteins have also been shown to use this pathway of endosome-TGN transport. Mannose 6-phosphate receptor (M6PR) and the small cation-dependent MPR46 might also travel through this pathway (Medigeshi and Schu, 2003; Lin et al., 2004) in addition to their recognized recycling pathway from the late endosome to the TGN, which is best defined by the recycling route of the TGN protease furin (Mallet and Maxfield, 1999). GLUT4, which is present in storage vesicles and is mobilized to the surface upon insulin stimulation to mediate glucose uptake, might also use this pathway to sort GLUT4 into the storage compartment for the next rounds of deployment to the surface (Bryant et al., 2002; Shewan et al., 2003). Consistently, BFLA1 and conA inhibit the generation of storage vesicles from the REs (Clague et al., 1994; Aniento et al., 1996). Parathyroid hormone (PTH)-related peptide (PTHrP) receptor might also recycle in a similar manner and its recycling is inhibited by BFLA1 and conA (Tawfeek and Abou-Samra, 2004), indicating a general role of V-ATPase in governing the sorting process at the peri-Golgi RE. Since other proteins such as P-selectin (Straley and Green, 2000), membrane-type matrix metalloproteinases (MT-MMPs) (Kang et al., 2002; Remacle et al., 2003; Wang, X. et al., 2004), copper transporters (Petris and Mercer, 1999; Petris et al., 2002) and sortilin (Nielsen et al., 2001) might also travel along this endosome-TGN pathway, BFLA1 and conA could be used to further dissect the trafficking pathway of these proteins.

The exact role of V-ATPase in RE sorting is not known but a potential function will be for V-ATPase to regulate VAMP4 so that the general traffic from REs to the TGN mediated by VAMP4 is indirectly influenced by V-ATPase. Support for this possibility includes the observation that physophilin/p39 of V-ATPase can be co-immunoprecipitated with antibody against VAMP4 (Steegmaier et al., 1999). Future experiments addressing the nature of the interaction of VAMP4 with

physophilin and the functional relevance of this interaction will be needed.

Both the SNARE complex consisting of syntaxin 16, syntaxin 6, Vit1a and VAMP3/4 (Mallard et al., 2002) as well as the SNARE complex consisting of syntaxin 5, Ykt6, GS28, and GS15 (Tai et al., 2004) have been shown to regulate traffic from the endosomes to the TGN. We have examined whether either or both of these SNARE complexes were involved in the recycling of VAMP4-EGFP by the knockdown of endogenous syntaxin 16 or GS15. The knockdown of either syntaxin 16 (Wang et al., 2005) (supplementary material Fig. S8) or GS15 (Tai et al., 2004) (supplementary material Fig. S9) had no major effect on the endocytosis and recycling of VAMP4-EGFP, suggesting that recycling of VAMP4 involves novel machinery. We believe that future studies along these lines will shed more light onto our understanding of membrane trafficking between endosomes and the TGN.

Materials and Methods

Materials

Normal rat kidney (NRK) cells were obtained from American Type Culture Collection. LipofectAMINE 2000 and G418 were purchased from Invitrogen. pEGFP-N1 vector was ordered from Clontech. Bafilomycin A1 (BFLA1), concanamycin A (conA), mouse-anti-FLAG and mouse-anti-β tubulin antibodies were purchased from Sigma. Nocodazole was obtained from Calbiochem. Vectashield mounting medium was from Vecto Laboratories. Rabbit antibody against GFP was purchased from Clontech. Rabbit anti-integrin alpha5 was purchased from Santa Cruz. Mouse antibody against GFP was from Roche. Monoclonal anti-EEA1, anti-rat transferrin receptor, anti-GM130, anti-syntaxin 6, anti-Vti1a, anti-GS15 and anti-clathrin heavy chain antibodies were obtained from Transduction Laboratories. Antibody against VAMP3 was from Affinity BioReagent. Fluorescein isothiocyanate (FITC)- or AlexaFluor 647 (AF647)-conjugated transferrin were from Molecular Probes. PE-, Cy3- or Cy5-conjugated secondary antibodies were from Jackson. Rabbit antibody against syntaxin 16 has been described previously (Mallard et al., 2002). Anti-LBPA antibody was a gift from Jean Gruenberg of University of Geneva, Switzerland. CHO-TacTGN38 stable cell line was a gift from Frederick R. Maxfield (Cornell University Medical College, New York, USA).

Expression constructs

VAMP4-EGFP, VAMP5-EGFP and V4nV5-EGFP constructs were previously described (Zeng et al., 2003). V4-EGFP/LL-AA was prepared using VAMP4-EGFP as a template. Two separate PCR products using oligonucleotides A (5′-GCTGAATTCCACCATGCCTCCCAAGTTCAAG-3′) and N1 (5′-TGAATCATCTTCCGCAGCATTTCTCCTCTC-3′) as well as N2 (5′-GAGAGGAGAAATGCTGCGGAAGATGATTCA-3′) and B (5′-GTGGATCCTTAGTACGGAATTTCACAACTATAAG-3′) were generated. The two PCR fragments were mixed and used as the template for a PCR using oligonucleotides A and B. The final PCR fragment was gel purified, digested with *Eco*RI and *Bam*HI, and ligated into the corresponding sites of pEGFP-N1. V4-EGFP/EDD-3A was prepared using VAMP4-EGFP as the template; two separate PCR products using oligonucleotides A and N3 (5′-TTCTTCATCTGATGCAGCTGCCAAAAGATTTCT-3′) as well as N4 (5′-AGAAATCTTTTGGCAGCTGCATCAGATGAAGAA-3′) and B were generated. The two PCR fragments were mixed and used as template for PCR with oligonucleotides A and B. The final PCR fragment was gel-purified, digested with *Eco*RI and *Bam*HI, and ligated into pEGFP-N1. V4-EGFP/5A was prepared using VAMP4-EGFP as the template; two separate PCR products using oligonucleotides A and N7 (5′-TAGAAAAAGGCCGCCGCTGCAGCTGAATCATC-3′) as well as oligonucleotides N8 (5′-GATGATTCAGCTGCAGCGGCGGCCTTTTTTCTA-3′) and B were generated. The two PCR fragments were mixed and then used as template for PCR using oligonucleotides A and B. The final PCR fragment was gel-purified, digested with *Eco*RI and *Bam*HI, and then ligated into pEGFP-N1. V4-EGFP/FF-AA was prepared using VAMP4-EGFP as the template; two separate PCR products using oligonucleotides A and N9 (5′-TGGTCCCCGTAGTGCAGCGTCCTCTTCTTC-3′) as well as oligonucleotides N10 (5′-GAAGAAGAGGACGCTGCACTACGGGGACCA-3′) and B were generated. The two PCR fragments were mixed and then used for PCR with oligonucleotides A and B. The final PCR fragment was gel-purified, digested with *Eco*RI and *Bam*HI, and then ligated into pEGFP-N1. The above constructs were each transfected into NRK cells using a LipofectAMINE-based system according to the protocol provided by the manufacturer as described previously (Zeng et al., 1998). Stable transfectants were selected by culturing the cells in medium with G418 (1000 μg/ml) and pooled transfectants were used for analysis.

Immunofluorescence microscopy

Cells grown on coverslips were washed twice with PBSCM (phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and then fixed in PBSCM containing 3% paraformaldehyde for 20 minutes. Fixed cells were washed twice with PBSCM, twice with PBSCM containing 50 mM NH_4Cl and then three times with PBSCM (5-10 minutes each). The cells were then permeabilized with 0.1% saponin in PBSCM and immunolabeled with respective primary and secondary antibodies as indicated. The coverslips were then washed three times with PBSCM containing 0.1% saponin and then rinsed twice with PBSCM. The cells were then mounted in Vectashield mounting medium. Confocal microscopy was performed with Zeiss AxioplanII microscope (Thornwood, NY) equipped with a Zeiss confocal-scanning laser.

Endocytosis of anti-EGFP antibody

Cells were incubated with rabbit anti-EGFP antibody (10 μg/ml) in DMEM at 37°C for 15-60 minutes. At the end of the incubation, cells were washed with cold PBSCM and subjected to fluorescence microscopy using Cy3-conjugated goat anti-rabbit antibody.

Kinetics of endocytosis and recycling

Cells were incubated at 4°C with either rabbit anti-EGFP antibody (10 μg/ml) alone or with both rabbit anti-EGFP and mouse anti-rat transferrin receptor antibodies (10 μg/ml) in cold DMEM for 1 hour. Unbound antibodies were removed by brief rinses using cold DMEM. The cells were then incubated at 37°C for different lengths of time in fresh DMEM. After incubation, fluorescence microscopy was performed.

Potassium depletion

Cells were washed twice with K^+-free buffer (140 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml of D-glucose, pH 7.4) and then incubated with hypotonic buffer (K^+-free buffer diluted 1:1 with water) for 5 minutes at 37°C. Cells were then washed twice with K^+-free buffer and then incubated in the same buffer for 30 minutes at 37°C followed by rabbit anti-EGFP in K^+-free buffer for 30-60 minutes before being fixed for immunofluorescence microscopy. As controls, parallel cultures were incubated in the same buffer supplemented with 10 mM KCl after hypotonic shock.

Hypertonic treatment

Cells were washed twice with hypertonic medium (DMEM supplemented with 0.45 M sucrose) and then incubated in the same medium for 30 minutes at 37°C. Rabbit anti-EGFP antibody was added and incubated for 30-60 minutes before being fixed for immunofluorescence microscopy. As a control, after hypertonic treatment, cells were incubated with antibody in DMEM.

Thermal perturbation of recycling

Cells were washed twice with cold DMEM and then incubated at 18°C for 15 minutes. Rabbit anti-EGFP antibody and/or fluorescent dye-conjugated transferrin were added followed by an additional incubation at 18°C for 60 minutes. As positive controls, parallel cultures were either incubated with antibody and/or ligand at 18°C or subjected to further incubation in fresh DMEM for 30 minutes at 37°C after the 18°C block.

Pharmacological perturbation of recycling

Cells were pre-incubated in culture medium alone or with 200 μM of chloroquine, 50 nM BFLA1 or 100 nM concanamycin A in DMEM at 37°C for 30 minutes. After the pre-incubation, rabbit anti-EGFP antibody and/or fluorescent dye-conjugated transferrin were added to the cells and incubated in the presence or absence of drugs for 30 minutes at 37°C before cells were fixed for immunofluorescence microscopy. As controls, parallel cultures were washed twice with DMEM after treatment and then subjected to a further 30-minute incubation in fresh DMEM in the absence of drugs at 37°C.

Nocodazole treatment

Cells were pre-treated with either 33 μM nocodazole or 0.1% DMSO as a control in DMEM for 30 minutes at 37°C. After the pre-treatment, cells were incubated with rabbit anti-EGFP antibody for 30-60 minutes at 37°C in the continuous presence of either nocodazole or DMSO alone.

Acidic stripping

Cells were incubated at 4°C with rabbit anti-EGFP antibody in cold DMEM for 1 hour. Unbound antibodies were removed by a brief rinse with cold DMEM. The cells were then incubated at 37°C for 15 minutes in fresh DMEM. Cells were then washed twice in cold acidic stripping buffer (0.2 M acetic acid, 0.5 M NaCl, 0.2% BSA, pH 2.5) to remove antibodies that remained on the surface (not internalized). After rinsing twice with cold PBSCM at 4°C, cells were incubated at 37°C for 30 minutes in fresh DMEM followed by fluorescence microscopy.

Identification of SNARE complex by co-immunoprecipitation

V4-EGFP-expressing NRK cells were incubated on ice with either 1 mM of NEM for 15 minutes, followed by 15 minutes with 2 mM DTT, or with 1 mM NEM quenched with 2 mM DTT for 30 minutes. Cells were then incubated for 30 minutes at 37°C before harvesting. Harvested cells were lysed in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1 mM PMSF and protease inhibitor cocktail). Lysates were incubated with either anti-GFP rabbit antibody or rabbit IgG at 4°C for 1 hour, followed by overnight incubation with TrueBlot anti-rabbit Ig IP beads at 4°C. Beads were washed five times with IP buffer and twice with IP buffer without Triton X-100 before immunoprecipitates were analyzed by western blotting for detecting the indicated proteins.

Flow cytometry

NRK cells or various EGFP-expressing stable pools were trypsinized and resuspended in PBS before being subjected to flow cytometry to detect and sort GFP-positive cells. To detect the level of cell surface GFP-fusion protein, NRK or stable pools expressing either VAMP4-EGFP or its various mutants was trypsinized and incubated with anti-GFP antibody on ice for 1 hour. After cold washing, cells were then incubated with PE-conjugated secondary antibody for 1 hour on ice before being subjected to flow cytometry.

Establishment of CHO cells expressing TacTGN38 and VAMP4-EGFP and co-trafficking assay

The construct expressing VAMP4-EGFP was transfected into CHO cells that stably express both human transferrin receptor and Tac-TGN38 (Ghosh et al., 1998; Mallet and Maxfield, 1999). A stable pool of transfectants were selected and used in the kinetic study. Cells were incubated with mouse monoclonal anti-Tac and rabbit anti-GFP for 1 hour at 4°C, followed by incubation in fresh medium at 37°C for different times. Cells were then fixed and subjected to immunofluorescence microscopy.

Inhibition of endocytosis using FLAG-tagged mutants of EPS15

Constructs expressing GFP-tagged mutants of Eps15 (GFP-EH29, GFP-DIII and GFP-D3Δ2) were kindly provided from Alexandre Benmerah (INSERM, Paris, France). These mutants were re-cloned into FLAG-tagged versions and then transiently expressed in HeLa cells or VAMP4-EGFP-expressing HeLa cells. Transfected cells were incubated with either Tf-AF555 alone or Tf-AF555 and rabbit-anti-GFP at 37°C for 30 minutes before being fixed, stained and analyzed using fluorescence microscopy.

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