Focal Exocytosis of VAMP3-containing Vesicles at Sites of Phagosome Formation

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Abstract. Phagocytosis involves the receptor-mediated extension of plasmalemmal protrusions, called pseudopods, which fuse at their tip to engulf a particle. Actin polymerizes under the nascent phagosome and may propel the protrusion of pseudopods. Alternatively, membrane extension could result from the localized insertion of intracellular membranes into the plasmalemma next to the particle. Here we show focal accumulation of VAMP3-containing vesicles, likely derived from recycling endosomes, in the vicinity of the nascent phagosome. Using green fluorescent protein (GFP) as both a fluorescent indicator and an exofacial epitope tag, we show that polarized fusion of VAMP3 vesicles precedes phagosome sealing. It is therefore likely that targeted delivery of endomembranes contributes to the elongation of pseudopods. In addition to mediating pseudopod formation, receptor-triggered focal secretion of endosomes may contribute to polarized membrane extension in processes such as lamellipodial elongation or chemotaxis.

Key words: cellubrevin • phagocytosis • macrophage • recycling endosomes • GFP

Introduction

Phagocytosis of microorganisms by leukocytes is a vital component of the host defense against infection. Microorganisms are initially coated by soluble host proteins called opsonins, which include fractions of complement and immunoglobulins. Subsequently, receptors on the surface of leukocytes recognize the opsonized microorganisms and initiate their phagocytosis. Engagement of opsonin receptors leads to their clustering along the surface of the phagocytic particle, followed by their tyrosine phosphorylation. Unlike most growth factor receptors, however, opsonin receptors, including those that recognize the Fc domain of immunoglobulin G (FcγR)[1], do not exhibit intrinsic tyrosine kinase activity. Instead, they are thought to be phosphorylated upon clustering by non-receptor kinases of the *src* family. Phosphorylation of tyrosine residues within the immunoreceptor tyrosine activation motif (ITAM), in turn provides docking sites for SH2-containing molecules, including the tyrosine kinase Syk (Greenberg et al., 1994). These early signaling events ultimately lead to local remodeling of the submembranous actin cytoskeleton (Greenberg et al., 1990), formation of membrane extensions called pseudopods and, finally, closure of the phagosome.

The accumulation of F-actin around the forming phagosome prompted the suggestion that force generated by the cytoskeleton propels the extension of the cell membrane, allowing its progressive receptor-mediated apposition or zippering onto the surface of the particle (Griffin et al., 1975). Though attractive, this model is likely incomplete, as it fails to account for the preservation of cell surface area during phagocytosis, especially in cases where multiple particles are internalized. Indeed, earlier studies have demonstrated that leukocytes can internalize an area equivalent to ~100% of their original membrane, with little or no net reduction in exposed membrane surface (Werb and Cohn, 1972). Moreover, flow cytometry determinations (Hackam et al., 1998), as well as estimates of plasmalemmal area by measurement of electrical capaci-

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[1]*Abbreviations used in this paper:* CHO-IIA, Chinese hamster ovary cells stably transfected with FcγRIIA receptors; EEA1, early endosome-associated antigen-1; FcγR, opsonin receptor that recognizes the Fc domain of immunoglobulin G; GFP, green fluorescent protein; ITAM, immunoreceptor tyrosine activation motif; LAMP1, lysosome-associated membrane protein 1; PM-GFP, membrane-targeted form of GFP; RBC-Ig, rabbit IgG-opsonized sheep RBC; TeTx, tetanus toxin; VAMP-Ig, chimera of VAMP3 and the constant region of the human IgG heavy chain.

tance (Holevinsky and Nelson, 1998) revealed that, rather than decreasing, the cell surface often increases during the course of phagocytosis. These results suggest that exocytosis of endomembranes accompanies phagocytosis, a conclusion consistent with the net increase in plasmalemmal area reported to occur during spreading of macrophages on IgG-coated surfaces, a process akin to abortive phagocytosis (Cox et al., 1999).

It is not clear if the putative exocytosis of endomembranes occurs at the time of phagocytosis, or whether it is a delayed compensatory response. It is similarly unclear whether exocytosis occurs randomly throughout the cell surface, or if it is instead targeted to the region of the nascent phagosome. Finally, the source of the endomembranes required to compensate for the area internalized remains unclear. In this regard, it was recently shown that the injection of tetanus toxin (TeTx) causes a decrease in the efficiency of phagocytosis (Hackam et al., 1998). In other systems, TeTx is known to inhibit exocytosis by catalyzing the proteolysis of certain isoforms of VAMP, a vesicle-associated fusion protein (Schiavo et al., 1992). We therefore speculated that compartments expressing TeTx-sensitive isoforms of VAMP would be likely to undergo exocytosis during phagosome formation. Among these, VAMP3 is most widely expressed and is predominantly localized to the recycling compartment of the early endosomes (McMahon et al., 1993; Daro et al., 1996). To test this prediction, and to analyze the spatial and temporal pattern of endomembrane delivery, we monitored the distribution of VAMP3 during the course of phagocytosis. For this purpose, we used antibodies raised to the endogenous VAMP3, as well as transfection of a chimeric construct of VAMP3 with GFP. Activation by a single, well-defined opsonin receptor was ensured by using Chinese hamster ovary cells stably transfected with FcγRIIA receptors (CHO-IIA cells). These cells not only recapitulate the phagocytic sequence (Indik et al., 1995), but are more amenable to transfection than native phagocytes.

Materials and Methods

Materials and Media

G418 sulfate and thapsigargin were from Calbiochem. Human IgG, 0.8 μm blue-dyed latex beads, and 3 μm latex beads were from Sigma. Sheep red blood cells (RBC) and rabbit anti-RBC IgG were from ICN-Cappel. Cy3-conjugated donkey anti–human IgG F(ab) fragment, anti–mouse IgG, anti–rabbit IgG, FITC-conjugated donkey anti–human IgG, and horseradish peroxidase-conjugated donkey anti–rabbit IgG were all from Jackson ImmunoResearch Laboratories. Rabbit anti-GFP IgG, the acetoxymethyl ester of 1,2-*bis*(aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (BAPTA-AM), and FM1-43 were from Molecular Probes. Mouse anti-LAMP1 antibody was from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa and John Hopkins University School of Medicine (Baltimore, MD).

DME and α-modified Eagle's medium (α-MEM) were obtained from Cellgro. Fetal bovine serum was from GIBCO and was heat-inactivated by incubation at 60°C for 30 min. Hepes-buffered bicarbonate-free RPMI 1640 medium was from Sigma. Ca^{2+}-free medium contained (in mM): 140 NaCl, 5 KCl, 5 glucose, 1 $MgCl_2$, 1 EGTA, and 20 Hepes (pH 7.4).

Constructs

To generate the VAMP3-GFP fusion protein construct, full-length rat VAMP3 was PCR-amplified from pCMV5-VAMP3 (provided by Dr. Thomas Sudhof, University of Texas Southwestern Medical Center, Dallas) using the oligonucleotide primers 5′-GAAGATCTCGCCACCATGTCTACAGGGGTGCCTTCAG-3′ and 5′-CCCAAGCTTAGAGACACACCACACAATGATG-3′. The cDNA was ligated into the BglII and HindIII sites of the pEGFP-N1 expression vector (Clontech). The construction of a vector comprising full-length VAMP3 fused to the CH2 and CH3 domains of the human IgG heavy chain (VAMP3-Ig) was described previously in Teter et al. (1998). A membrane-targeted form of GFP (PM-GFP, provided by Dr. Tobias Meyer, Duke University) was engineered by fusing the NH_2-terminal 10–amino acid acylation sequence of Lyn to GFP (Teruel et al., 1999).

Cell Culture and Transfection

Chinese hamster ovary cells stably transfected with FcγRIIA receptors (CHO-IIA) were maintained in DME supplemented with 10% fetal calf serum and 1 mg/ml G418. Chinese hamster lung fibroblasts stably transfected with FcγRIIA receptor tagged at its COOH terminus with enhanced GFP were maintained in α-MEM supplemented with 10% fetal calf serum and 1 mg/ml G418. The murine cell line J774 was maintained in DME with 10% fetal calf serum. All cell lines were maintained at 37°C under 5% CO_2.

Where indicated, cells grown on 25-mm glass coverslips were transiently transfected with cDNAs encoding either VAMP3-GFP, PM-GFP, or VAMP3-Ig. In all cases the cells were transfected using the Fugene-6 reagent (Boehringer Mannheim) as suggested by the manufacturer and used 2 d after transfection.

Phagocytosis

CHO-IIA cells were incubated at 37°C with either rabbit IgG-opsonized sheep RBC (RBC-Ig) or human IgG-opsonized latex beads (3 μm; Sigma) for 10 min. Excess particles were washed away with PBS and the cells were incubated in DME at 37°C for additional times. To identify adherent RBC-Ig or opsonized latex beads that were not internalized, the samples were incubated at 4°C in Hepes-buffered medium containing Cy3-labeled donkey anti–rabbit IgG (1:1,500) or FITC-labeled donkey anti–human IgG (1:1,000) for 40 min.

Where indicated, calcium transients during phagocytosis were precluded by preloading the cells with BAPTA by incubation with 10 μM of the parent acetoxymethyl ester for 20 min at 37°C in Ca^{2+}-free medium, followed by depletion of the intracellular stores by addition of 100 nM thapsigargin for 10 min at 37°C.

To assess changes in cell surface area during phagocytosis, CHO-IIA cells which had internalized IgG-opsonized latex beads were trypsinized, washed several times with ice-cold PBS, and resuspended at 10^6 cells/ml. Control cells were not exposed to particles. Cells were then incubated with 1 μM FM1-43 for 5 min on ice to label the cell surface (Betz and Bewick, 1992), and FM1-43 fluorescence was quantified by flow cytometry. Cells containing latex beads were identified by their increased side scatter.

Fluorescence Labeling and Analysis

To identify early endosomes, cells transfected with VAMP3-GFP were serum-starved for 1 h in DME, followed by incubation in serum-free DME containing 25 μg/ml tetramethylrhodamine-labeled transferrin for 1 h at 37°C. Labeling of surface VAMP3-Ig was accomplished by incubating transfected cells for 40 min at 4°C in Hepes-buffered medium containing Cy3-labeled anti–human IgG F(ab) fragment (1:1,000). Extracellular VAMP3-GFP was labeled by incubation at 4°C in Hepes-buffered medium containing rabbit anti-GFP (1:10,000) for 40 min, followed by fixation with 4% paraformaldehyde for 1 h and addition of Cy3-conjugated secondary antibody. To localize the lysosome-associated membrane protein-1 (LAMP1), cells were fixed with −20°C methanol for 15 min, labeled with mouse anti-LAMP1 for 40 min, followed by a Cy3-conjugated secondary antibody. Fluorescence images were acquired on a DMIRB Leica microscope with a Micromax cooled CCD camera (Princeton Instruments).

Phagosome Isolation

Phagosomes were isolated by the method described in Desjardins et al. (1994). In brief, J774 cells were grown on 14-cm petri dishes to 80–90% confluence. Blue-dyed latex beads were added and the mixture was incubated at 37°C for 10 or 60 min. The cells were then washed with ice-cold PBS, resuspended in homogenization buffer (250 mM sucrose, 3 mM imidazole, plus protease inhibitors) and lysed using a Dounce homogenizer.

Unbroken cells were removed and the lysate was mixed with a 60% sucrose, 3 mM imidazole stock (pH 7.4) to obtain a 40% sucrose-imidazole solution, which became part of a discontinuous gradient consisting of 10, 25, 35, 40, and 60% sucrose-imidazole steps. The gradient was centrifuged at 100,000 *g* for 60 min and the phagosome fraction was collected from the 10–25% interphase. After washing in PBS, the protein concentration of the phagosomal preparation was determined using the bicinchoninic acid assay (BCA; Pierce), using BSA as a standard.

SDS-PAGE and Immunoblotting

Samples were solubilized in Laemmli's sample buffer, resolved by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. Membranes were blocked overnight with 5% milk in PBS and 0.05% Tween 20 and then incubated with affinity-purified rabbit antibodies to the early endosome antigen-1 (EEA1, 1:1,000, provided by Dr. Marino Zerial, European Molecular Biology Laboratory, Heidelberg, Germany), to the 39-kD subunit of the V-ATPase (1:2,000), or to VAMP3 (1:100) for 1 h in PBS-Tween containing 1% serum albumin. The blots were then washed in PBS-Tween, followed by a 1-h incubation with horseradish peroxidase–conjugated donkey anti–rabbit IgG at 1:2500. Finally, the membranes were washed and developed using enhanced chemiluminescence (Amersham).

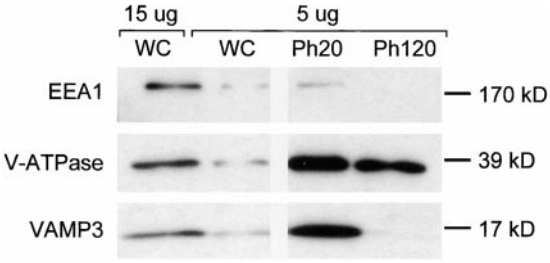

Figure 1. VAMP3 is enriched in early phagosomes. J774 cells were allowed to internalize latex beads for either 20 min or 2 h and phagosomes were purified by gradient centrifugation. Samples of whole cell lysates (WC) or of phagosomes formed after 20 min (Ph20) or 2 h (Ph120) were resolved by SDS-PAGE and immunoblotted with polyclonal antibodies against EEA1 (top), the 39-kD subunit of the V-ATPase (middle), and VAMP3 (bottom). The amount of protein loaded in each lane is indicated at the top. Representative of at least three similar experiments.

Results

VAMP3 Is Present in Phagocytes and Becomes Enriched in Phagosomal Membranes

To determine the TeTx-sensitive isoforms of VAMP expressed in phagocytic cells we examined total cell lysates from two macrophage cell lines, J774 and RAW264.7. Western blotting revealed that these cells expressed comparatively little VAMP2, while VAMP3 was abundantly expressed (not illustrated). Therefore, we confined our studies to the distribution of VAMP3 during phagocytosis.

The available antibodies to VAMP3 were not suitable for analysis of subcellular distribution by immunofluorescence. Instead, we analyzed the association of VAMP3 with phagosomes by a combination of cellular fractionation and immunoblotting. J774 cells were allowed to internalize latex beads, which were subsequently purified by flotation on density gradients (Desjardins et al., 1994). As illustrated in Fig. 1, VAMP3 was greatly enriched in the early phagosomal membrane relative to a whole cell lysate. The early endosomal membrane marker EEA1 (Mu et al., 1995), was also detectable in early phagosomes, but was not noticeably enriched compared with the cell lysate. Expectedly, the vacuolar ATPase was also enriched in the phagosome fraction (Fig. 1), consistent with earlier findings (Pitt et al., 1992). Two hours after phagocytosis, the V-ATPase remained enriched in the phagosomes, but both VAMP3 and EEA1 had been largely depleted from the phagosomal membrane (Fig. 1).

VAMP3-GFP Localizes to the Recycling Endosomes

While the preceding experiments indicate that VAMP3 accumulates in early phagosomes, they provide little information regarding the precise site and time of incorporation of the vSNARE into the phagosomal membrane. Because isolation of purified membranes from phagosomes at earlier stages of formation is not feasible, we opted instead to follow the distribution of VAMP3 during particle internalization by noninvasive means, monitoring the fluorescence of GFP. A VAMP3-GFP chimeric construct was transfected into CHO-IIA cells, which were validated earlier as good models for the study of phagosomal formation and maturation (Indik et al., 1995; Downey et al., 1999). Unlike phagocytes, these cells are readily transfectable and are comparatively large and flat, facilitating the subcellular localization of the probe.

The distribution of VAMP3-GFP was monitored by fluorescence microscopy and compared with that of rhodamine-labeled transferrin. As shown in Fig. 2, a and b, VAMP3-GFP accumulates predominantly in juxtanuclear early endosomes, likely the recycling subcompartment, as described earlier for endogenous VAMP3 (McMahon et al., 1993; Daro et al., 1996). Because VAMP3 is a type II protein, its COOH terminus is expected to reside in the lumen of the endosomes and to be exposed to the extracellular milieu while on the plasma membrane. We took advantage of this feature to ascertain that the heterologously expressed protein is properly oriented and, more importantly, that it is able to recycle between endomembranes and the plasmalemma. CHO-IIA cells were cotransfected with VAMP3-GFP and VAMP3-Ig. To assess the partition of VAMP3 between the plasma membrane and recycling endosomes, cells were exposed to Cy3-labeled antibody against human IgG at 4°C to label only exofacial VAMP3. Monovalent F(ab) fragments of the antibody were used to preclude aggregation and possible mistargetting. It is noteworthy that, while the fraction of plasmalemmal VAMP3 can be readily detected by this method (Fig. 2 d), the majority of the vSNARE is located intracellularly. Indeed, when visualized by the fluorescence of GFP in the same cells, the plasmalemmal component of VAMP3 is barely detectable (Fig. 2 c, see also Fig. 2, a and e). When cells prelabeled with the F(ab) fragments were subsequently rewarmed to 37°C for 1 h to re-initiate membrane traffic, the conjugated VAMP3-Ig was rapidly internalized and its pattern merged with that of VAMP3-GFP (Fig. 2, e and f). Jointly, these observations indicate that chimeric forms of VAMP3 target predominantly to early endosomes, where they appear to be competent for recycling.

Redistribution of VAMP3-GFP during Phagocytosis

The fate of VAMP3 during phagocytosis was analyzed next

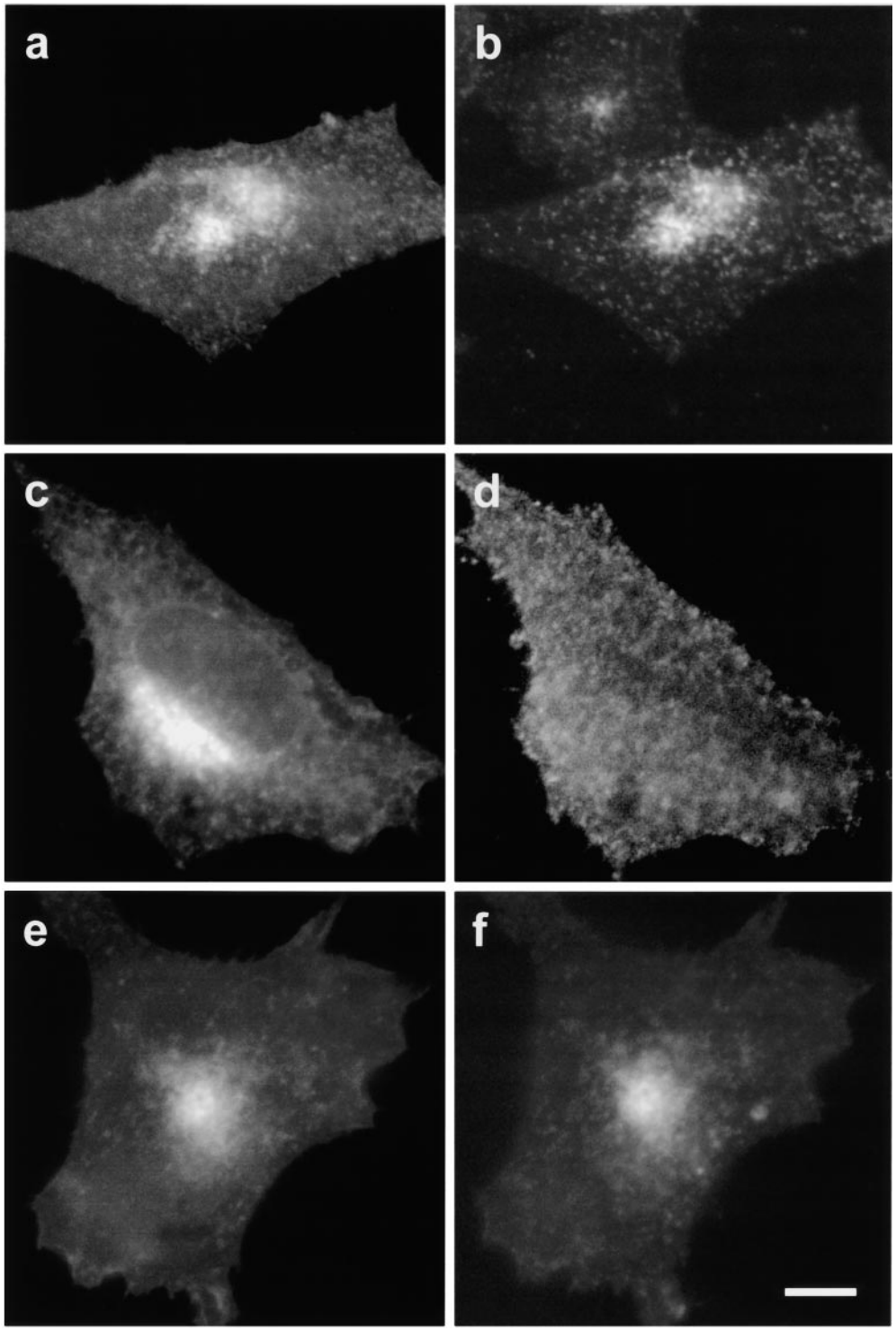

Figure 2. Localization and recycling of VAMP3 in CHO-IIA cells. (a and b) CHO-IIA cells transfected transiently with VAMP3-GFP were incubated with 25 μg/ml tetramethylrhodamine-labeled transferrin for 1 h at 37°C, washed and visualized by fluorescence microscopy. (a) VAMP3-GFP (green fluorescence); (b) transferrin (red fluorescence). (c–f) CHO-IIA cells transiently cotransfected with VAMP3-GFP and VAMP3-Ig were incubated at 4°C with Cy3-labeled F(ab) fragments of antibodies raised against human IgG, to label exofacial VAMP3-Ig. In c and d the cells were visualized at this stage. In e and f, the cells were next warmed to 37°C and incubated for an additional h to allow internalization of the labeled VAMP3-Ig. (c and e) VAMP3-GFP (green fluorescence); (d and f) Cy3-labeled VAMP-Ig (red fluorescence). Representative of at least three similar experiments. Bar, 10 μm.

by transient transfection of CHO-IIA cells. Formation of phagosomes was induced by exposure of the transfectants to RBC-Ig. Cy3-labeled antibodies to the opsonizing IgG were used to assess the location of the particles. As illustrated in Fig. 3 a, a redistribution of VAMP3-GFP occurred shortly after exposure to RBC-Ig. At early times (≤10 min) VAMP3 was found to demarcate the periphery of forming phagosomes. The accumulation of VAMP3-GFP around the RBC-Ig appeared to precede internalization of the particles, as indicated by the fact that the RBC-Ig remained accessible to anti-IgG antibodies added extracellularly (Fig. 3 b). As time proceeded, the particles became fully internalized and therefore inaccessible to the antibody (Fig. 3 d). Concomitantly, the amount of VAMP3-GFP associated with the phagosome decreased (Fig. 3 c), becoming undetectable by 90 min (Fig. 3, e and f). Together, these observations indicate that VAMP3-containing endomembranes become transiently associated with the phagosome.

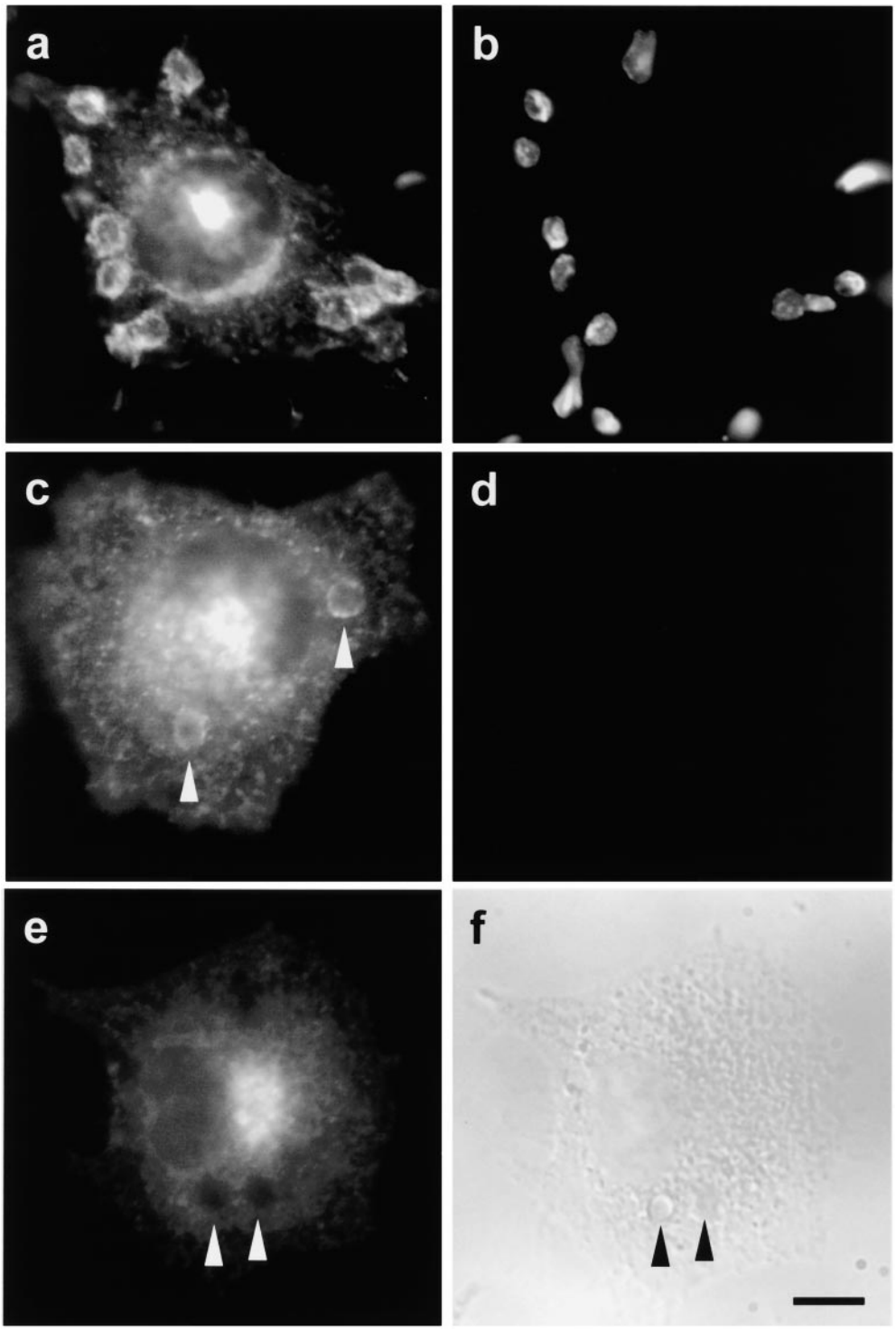

Figure 3. Localization of VAMP3-GFP in CHO-IIA cells during phagocytosis. CHO-IIA cells transfected with VAMP3-GFP were incubated at 37°C with RBC-Ig for 10 (a and b), 30 (c and d), or 90 min (e and f). The distribution of VAMP3-GFP (green fluorescence) is shown in a, c, and e. In b and d, extracellular RBC-Ig were detected by incubation at 4°C with Cy3-labeled antibodies against the opsonizing IgG. f shows the differential interference contrast image of the cell in e. Arrowheads indicate internalized RBC-Ig. Representative of at least three similar experiments of each type. Bar, 10 μm.

Increased Surface Exposure of VAMP3 during Phagocytosis

Accumulation of the fluorescent protein in the vicinity of nascent phagosomes indicates mobilization of VAMP3 to the cell periphery, but does not provide evidence that exocytosis occurred at this site. To evaluate this possibility, we monitored the extracellular appearance of the COOH terminus of the chimera using an antibody against GFP. CHO-IIA cells transiently transfected with VAMP3-GFP were allowed to interact with opsonized particles for 10 min and, after cooling to 4°C, the exposure of GFP to externally added primary and secondary antibodies was examined. As before, VAMP3-GFP accumulated around forming phagosomes (Fig. 4 a). Importantly, the COOH-terminal domain of the chimera was exposed to the external medium, as attested by its accessibility to anti-GFP an-

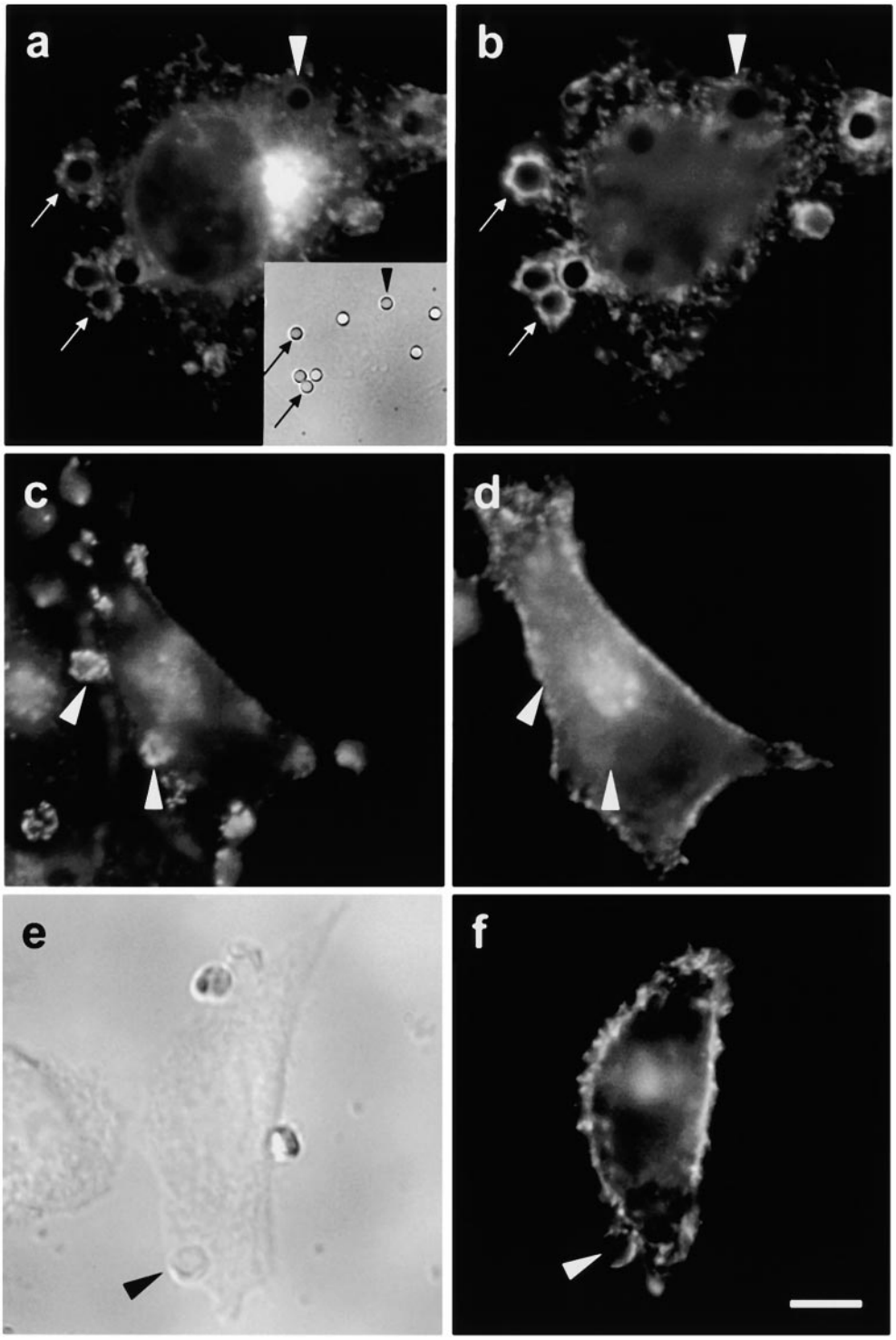

Figure 4. Surface exposure of VAMP3-GFP in CHO-IIA cells during phagocytosis. (a–b) CHO-IIA cells transfected with VAMP3-GFP were incubated at 37°C with IgG-opsonized latex beads for 10 min. After stopping phagocytosis by cooling to 4°C, exofacial VAMP3-GFP was detected by incubation with antibodies to GFP followed by secondary Cy3-conjugated antibodies. (a) Distribution of VAMP3-GFP (green fluorescence). Arrows indicate beads that are undergoing phagocytosis, whereas the arrowhead indicates a bead not being internalized. Inset shows the corresponding bright field image. (b) Distribution of exofacial VAMP3-GFP (red fluorescence). (c and d) Cells stably expressing FcγRIIA-GFP were transiently transfected with VAMP3-Ig and then incubated at 4°C with Cy3-labeled F(ab) fragments of antibodies raised against human IgG, to label exofacial VAMP3-Ig. Finally, opsonized RBC were added to induce receptor clustering. (d) Distribution of FcγRIIA-GFP (green fluorescence). Arrowheads point to FcγRIIA-GFP clusters formed at sites of interaction with the RBC. (d) Distribution of VAMP3-Ig (red fluorescence). (e and f) CHO-IIA cells were transiently transfected with PM-GFP. Next, RBC-Ig were added to initiate phagosome formation. (e) Bright field image. Arrowhead indicates a nascent phagosome. (f) Distribution of PM-GFP cell in (e). Representative of at least three experiments of each type. Bar, 10 μm.

tibodies (Fig. 4 b). Note that under these conditions the remaining juxtanuclear VAMP3-GFP remains inaccessible to the antibody.

The marked enrichment of VAMP3-GFP in the area of the plasmalemma subjacent to the opsonized particle suggests that focal exocytosis of recycling endosomes occurred at the site of phagosome formation. However, because a fraction of the VAMP3 is present constitutively at the plasma membrane, accumulation underneath the phagosome could also have occurred as a result of lateral diffusion and focal immobilization of the chimera. Indeed, as mentioned in the Introduction, such lateral mobilization or capping is responsible for the enrichment of Fc receptors in the phagosomal cup. We therefore compared the lateral displacement of FcγRIIA receptors and of plasmalemmal VAMP3 during the course of particle binding and cup formation. Chinese hamster cells were co-transfected with a GFP-tagged FcγRIIA receptor and with

VAMP3-Ig. The exofacial VAMP3-Ig was then labeled with Cy3-conjugated F(ab) fragments at 4°C before phagocytosis. Under these conditions, both the FcγRIIA receptors and the labeled VAMP3-Ig are homogeneously distributed on the plasma membrane (not illustrated). Upon exposure to opsonized particles, accumulation of FcγRIIA receptors in phagosomal cups was readily apparent (Fig. 4 c). In contrast, no accumulation of the prelabeled VAMP3 was detected under these conditions (Fig. 4 d). These findings suggest that the concentration of exofacial VAMP3-GFP in the vicinity of the phagosome reported in Figs. 3 and 4 is not likely due to lateral accumulation of molecules present at the surface before phagocytosis.

It may be argued that rather than resulting from the targeted exocytosis of endomembranes, the enhanced VAMP3-GFP signal at nascent phagosomes merely reflects an increase in the overall membrane density in the region of the ingested particle, perhaps resulting from localized ruffling. To examine this possibility, we monitored the fluorescence of PM-GFP. Upon acylation, promoted by attachment of the 10 NH_2-terminal amino acids of Lyn (Teruel et al., 1999), GFP acquires the tendency to bind predominantly to the inner leaflet of the plasmalemma (Fig. 4 f). In cells exposed to RBC-Ig, PM-GFP followed the contour of the phagosomal cup, but failed to show the marked accumulation reported above for VAMP3-GFP. Thus, localized ruffling of the plasmalemma cannot explain the accumulation of VAMP3 in the region of the nascent phagosome.

Change in Cell Surface Area during Phagocytosis

To further verify that the exocytosis of endomembranes accompanies phagocytosis, we examined the change in cell surface area during particle ingestion. CHO-IIA cells were initially allowed to ingest opsonized latex beads. Under the conditions used, each of the phagocytically active cells ingested five or more beads. Considering the size of the latex beads used (3 μm in diameter) an area equivalent to 27% of the original surface area of CHO-IIA cells was internalized (calculated assuming 5 beads/cell and a cell surface area of ~530 μm^2, based on a spherical cell diameter of 13 μm). To estimate the effective change in surface area accompanying this ingestion, we used the dye FM1-43, which becomes fluorescent when intercalated into the outer leaflet of the plasma membrane (Betz and Bewick, 1992). Cells with and without internalized particles were detached, cooled on ice and incubated with FM1-43. Flow cytometry analysis of FM1-43 fluorescence revealed that cells with internalized particles, identified by the increase in their side scatter relative to control cells, had an average fluorescence increase of ~35%. The enhanced fluorescence, which reflects increased surface area after phagocytosis, further supports the notion that endomembranes are inserted into the plasmalemma during this process, consistent with the appearance of VAMP3 at sites of phagosome formation.

VAMP3 Accumulation Does Not Require Calcium

Cross-linking of Fc receptors has been shown to increase free cytosolic calcium in phagocytes (Jaconi et al., 1990; Odin et al., 1991). In addition, elevation of cytosolic calcium upon addition of ionophores was shown earlier to accelerate the exocytosis of endosomes in murine macrophages (Buys et al., 1984). It was therefore conceivable that localized changes in calcium triggered the focal exocytosis of VAMP3-containing membranes during FcγR-mediated phagocytosis. To test this notion, CHO-IIA cells were loaded with BAPTA and pre-treated with thapsigargin in calcium-free medium, in order to deplete the calcium stored in the endoplasmic reticulum. When subsequently exposed to opsonized particles, the localized accumulation of VAMP3-GFP in the vicinity of nascent phagosomes was unaffected (not shown). These findings are in accordance with the observation that, in macrophages, phagocytosis is calcium independent (Di Virgilio et al., 1988).

Distribution of LAMP1 during Phagocytosis

Recruitment and exocytosis of lysosomes was shown to be required for *Trypanosoma cruzi* invasion of mammalian cells (Tardieux et al., 1992). To evaluate whether lysosomes are also secreted in the vicinity of the forming phagosome, we analyzed the distribution of LAMP1, a late endosomal/lysosomal marker. Unlike the early and transient interaction found above for VAMP3, the LAMP1-containing compartment was not mobilized to the area of the nascent phagosome (Fig. 5, a and b). Instead, LAMP1-containing vesicles merged with the phagosome only at later stages of maturation, becoming clearly detectable after 60 min (Fig. 5, c and d). Therefore, insertion of LAMP1-containing vesicles is unlikely to contribute significantly to the increase in surface area observed upon phagocytosis.

Discussion

We had previously shown that phagocytosis by J774 and CHO-IIA cells could be partially inhibited by microinjection or transfection of TeTx (Hackam et al., 1998), suggesting that a VAMP-dependent vesicle fusion step is important for phagosome formation. In this study we have shown that the TeTx substrate VAMP3 is targeted to the nascent phagosome, where it is secreted focally before closure of the phagosomal membrane. VAMP3 is a primary component of the recycling endocytic compartment and has been implicated in the delivery of transferrin receptors to the cell surface (Galli et al., 1994). Early endosomes had been shown earlier to deliver transferrin receptors to formed phagosomes during the course of their maturation (Sturgill-Koszycki et al., 1996). Our studies indicate that vesicles derived from the recycling compartment are important not only in the maturation of phagosomes, but also in their formation.

The concept that membrane addition may be required for phagocytosis has only recently come to the fore, as previous models had proposed that the internalization of particles resulted from the direct apposition of the cell membrane to the surface of the particle via opsonin receptors by a zippering action (Griffin et al., 1975), followed by contractile closure by a purse-string contractile mechanism (Swanson et al., 1999). By themselves, however, these combined hypotheses would predict a net loss of cell sur-

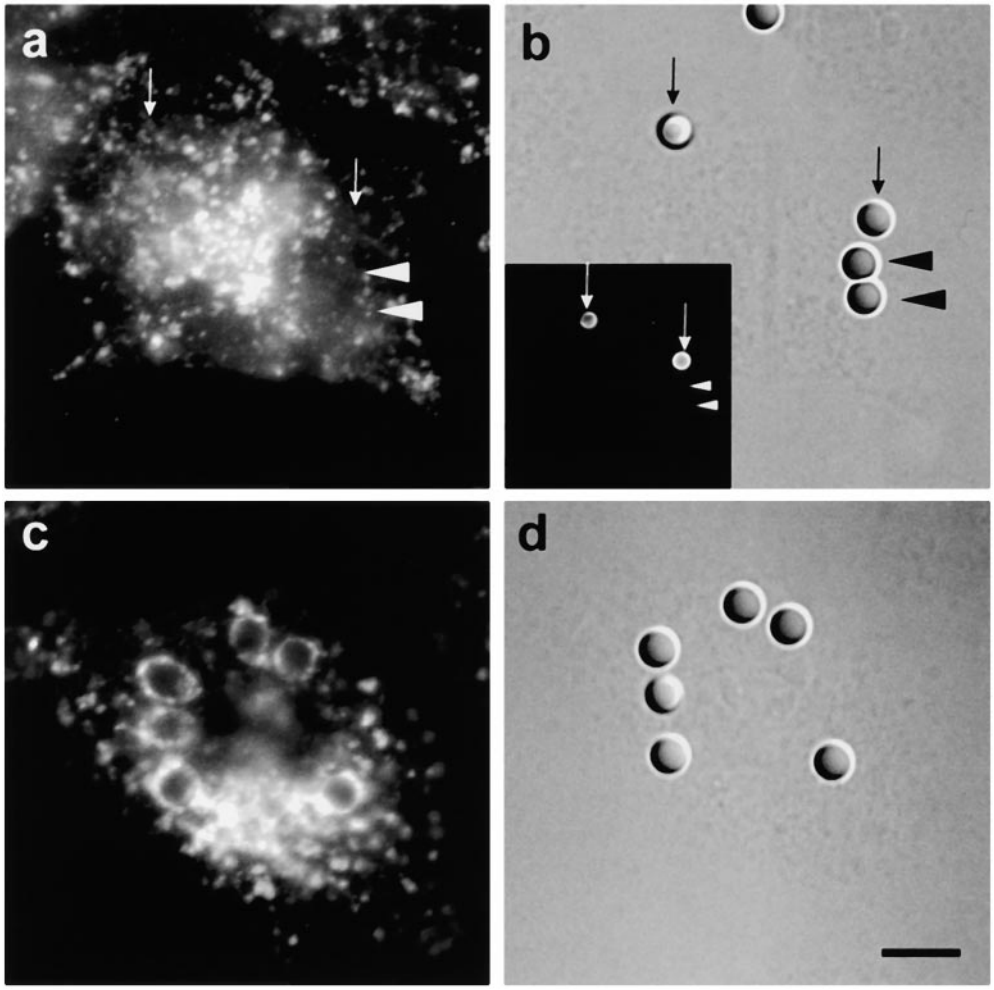

Figure 5. Localization of LAMP1 in CHO-IIA cells during phagocytosis. CHO-IIA cells were incubated at 37°C with IgG-opsonized latex beads for 10 min (a and b) or 60 min (c and d). The distribution of LAMP1, detected by indirect immunofluorescence using Cy3-labeled antibodies, is shown in a and c. b and d show the differential interference contrast images of the cells in a and c, respectively. In a and b, arrowheads indicate beads that have been internalized, whereas arrows indicate extracellular beads, identified by accessibility to extracellularly added labeled antibodies. (Inset) Extracellular particles were detected by incubation at 4°C with FITC-labeled antibodies against the opsonizing IgG. Bar, 10 μm (does not apply to inset).

face area during phagocytosis, particularly when large and/or multiple particles are ingested. In actuality, electrophysiological studies have demonstrated a transient but rapid increase in membrane capacitance during the signaling phase of phagocytosis, followed by stepwise decreases that correspond with particle internalization (Holevinsky and Nelson, 1998). In addition, studies in human neutrophils revealed that externalization of CD63 and CD66b, indicative of exocytosis of primary and secondary granules, respectively, preceded closure of the phagosome (Tapper and Grinstein, 1997). So, although the accumulation of F-actin around the nascent phagosome (Greenberg et al., 1990) has led many to speculate that the growth of pseudopods around the particle is driven by the extension of actin filaments, it may be mediated, at least in part, by the focal delivery of endomembranes to the area of phagosome formation. The increase in surface area would permit the receptor- and/or actin cytoskeleton-driven zippering of the membrane around the particle. Therefore, we regard endomembrane insertion not as an alternative but as complementary to the zippering model.

We can only speculate as to how the polarized insertion of VAMP3-containing vesicles is achieved. In yeast, Cdc42 is required for polarized bud growth (Adams et al., 1990), and its deletion in MDCK epithelial cells results in the selective inhibition of membrane traffic to the basolateral membrane (Kroschewski et al., 1999). Interestingly, Massol et al. (1998) have found that in RBL-2H3 cells, inhibition of Cdc42 blocks IgE-mediated phagocytosis. Although in these experiments the attached particles were found on actin-rich, pedestal-like structures, there was no apparent pseudopod extension, suggesting that Cdc42 may in fact mediate the extension of membrane over the particle edges by targeting vesicles to these sites, ultimately allowing phagocytosis to occur. How the endomembranes are focally targeted to a restricted area on the plasmalemma also remains obscure, but the sec6/8 complex, which is important for polarized membrane addition in yeast (TerBush et al., 1996) and epithelial cells (Grindstaff et al., 1998), may similarly be involved in the recruitment of vesicles to sites of phagocytosis.

Our observation that phagocytosis is mediated by the localized growth of membranes through targeted delivery of recycling endosomes may have broader implications. Many cellular processes, such as the formation of lamellipodia, macropinocytosis, cell spreading and chemotaxis, as well as bacterial invasion, all result from receptor signaling that leads to localized expansion of the plasma membrane. While actin polymerization has been implicated in each of these processes, growing evidence supports the notion that membrane addition may also be required (Bretscher, 1996). The recycling endosome compartment may provide

an ideal source for such localized growth; as it represents a large reservoir of membrane area, it contains the targeting elements required for fusion with the plasma membrane and is also capable of interacting with microtubules. Vectorial traffic along microtubules may contribute to the polarized delivery to the site of receptor activation. Fluorescent forms of VAMP3 should prove useful in testing the role of recycling endosomes in other biological responses.

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