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Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation

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SUMMARY

Endocytosis of the Tf/TfR complex is essentially the only pathway active in maturing reticulocytes, while exosomes, formed by invagination of the endosomal membrane, provide a mechanism to eliminate seemingly obsolescent proteins, including the TfR, when their function is completed. In this study, we examined molecular trafficking in the recycling and exosome-directed pathways during endocytosis in maturing reticulocytes. To this end, the flow of two exogenously inserted fluorescent lipid analogs, *N*-(*N*-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]) sphingomyelin (C6-NBD-SM) and *N*-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE) was monitored and compared to that of the transferrin (Tf)/Tf receptor (TfR) complex. Prior to elimination via exosomes, the TfR actively recycles with a half-time of approx. 2 minutes. The recycling kinetics of C6-NBD-SM, as bulk plasma membrane marker, are identical to those of the apoTf/TfR complex, as shown by fluorescence microscopy and biochemical analysis. By contrast, although efficiently internalized along the same pathway, N-Rh-PE does not return to the cell surface. More specifically, sucrose gradient analysis and immunoisolation experiments demonstrated that N-Rh-PE accumulates in exosomes, which are eventually released into the extracellular medium. Fluorometric measurements showed that exogenously inserted N-Rh-PE is present in the reticulocyte plasma membrane as small molecular clusters. Moreover, a close correlation was observed between the fate of crosslinked proteins, including the TfR and acetylcholinesterase (AChE), and the fate of the clustered lipid N-Rh-PE. Thus antibody-induced aggregation of specific proteins like the TfR and AChE, which are normally sorted into exosomes during reticulocyte maturation, enhances their shedding by the exosomal pathway. Taken together, the results support the hypothesis that aggregation of either proteins or lipids act as a general sorting signal for exosomal processing, thereby inhibiting reentry in a recycling pathway and providing an effective means for clearing molecules from the cell surface and their eventual elimination from the cells.

Key words: Aggregation, C6-NBD-SM, Endocytosis, Exosome, N-Rh-PE, Reticulocyte, Sorting

INTRODUCTION

Upon its maturation into an erythrocyte, the reticulocyte loses several obsolescent membrane-associated activities, such as e.g. the ability to bind transferrin (Tf) and the capacity to transport glucose. The functional proteins involved are released into the extracellular medium following initial internalization and packaging into small membrane vesicles, termed exosomes (Johnstone et al., 1987), which are derived from multivesicular endosomes (MVE). These structures are formed after budding and pinching off of the endosomal membrane into the lumenal space (Harding et al., 1985; Davis et al., 1986; Pan et al., 1985). In this context, it is of interest to note that it has been suggested that the formation of similar multivesicular bodies (MVB) in endosomes in other cell types provides a means for lysosomal delivery of proteins destined for degradation (van Deurs et al., 1993). In the case of reticulocytes, exosomes are released into the extracellular medium which occurs when the MVEs fuse with the plasma membrane (Fig. 1). However, the underlying mechanism for elimination of obsolescent proteins in maturing reticulocytes is entirely obscure. Presumably, the proteins involved have to be sorted into domains of the endosomal membrane that will form intralumenal vesicles.

Membrane flow during endocytosis can be conveniently monitored when using fluorescently tagged lipid analogs (Koval and Pagano, 1991; Hoekstra and Kok, 1992). Fluorescently labeled sphingomyelin (*N*-(*N*-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl])-sphingomyelin; C6-NBD-SM) is efficiently recycled to the plasma membrane after endocytosis according to a bulk flow mechanism. This recycling event is kinetically and morphologically identical to that of fluorescently labeled Tf (Mayor et al., 1993). Hence, it would appear that the recycling of distinct receptors, such as the Tf-receptor (TfR), would not require (a) specific signal(s). The implication of this suggestion would then be that sorting signals must, however, exist to target membrane components from early endosomes to lysosomes (Mayor et al., 1993).

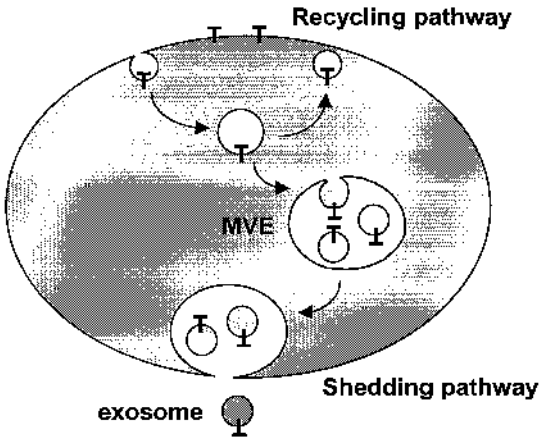

Fig. 1. TfR pathways during reticulocyte maturation. TfR (T) are internalized at the cell surface and then either recycled back to the plasma membrane (recycling pathway) or segregated in small vesicles constituting multivesicular endosomes (MVE). The receptor is expelled into the extracellular medium, as part of an exosome, when MVE fuse with the plasma membrane (shedding pathway).

Molecular aggregation could represent such a signal since membrane components that normally recycle can be rerouted towards the lysosomal pathway in an aggregated form (Mellman and Plutner, 1984; Weismann et al., 1986). Interestingly, *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (N-Rh-PE), after insertion into the plasma membrane of baby hamster kidney cells, has been shown to be selectively sorted from other fluorescent lipid analogs and is targeted to lysosomes (Kok et al., 1990). Since N-Rh-PE is presumably present in the membrane in small molecular clusters, it has been proposed that such clustering triggers lysosomal targeting.

In reticulocytes, the endocytic pathway is only partially operative, its sole purpose being to ensure the intracellular delivery of Fe via the Tf/TfR complex. Since lysosomes are almost absent, exosomal secretion is considered as the final step of the endocytic pathway. However, it has been shown recently that exosomal processing is not unique to reticulocytes, but may also play a role during antigen presentation (Raposo et al., 1996). Hence, a detailed study of exosomal processing per se may well bear a broader physiological significance than anticipated thus far.

In this paper we describe the fate of two fluorescent lipid analogs C6-NBD-SM and N-Rh-PE, after their insertion in the plasma membrane of reticulocytes. We demonstrate that the two lipid analogs are very convenient membrane markers for the two pathways chronologically followed by the TfR in maturing reticulocytes. Evidence is presented, showing that C6-NBD-SM is transported through the recycling pathway, the route followed by the TfR when the cell is still in demand of iron. Interestingly, N-Rh-PE is rapidly sorted and targeted into exosomes. In conjunction with observations of a rerouting of proteins, including the Tf/TfR complex and the GPI-anchored AChE upon their aggregation, our results strongly support the view that molecular clustering of either proteins or lipids within the plane of the membrane represents a sorting signal, which programs a cellular clearing mechanism via either exosomes (reticulocytes) or lysosomal digestion (other cell types).

MATERIALS AND METHODS

Materials

N-(*N*-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl])-sphingomyelin (C6-NBD-SM) and *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (N-Rh-PE) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Dioleoylphosphatidylethanolamine (DOPE), human transferrin (Tf), anti-human Tf, rabbit anti-mouse IgG, rabbit anti-goat IgG, sheep anti-rabbit IgG and alkaline phosphatase conjugated with goat anti-mouse IgG antibodies were obtained from Sigma (St Louis, MO). A mouse monoclonal antibody against rat transferrin receptor was purchased from Chemicon (Temecula, CA). A peroxidase-conjugated sheep anti-mouse IgG antibody was obtained from the Pasteur Institute (Paris, France). Prestained molecular mass standards were from Sigma (St Louis, MO) and Bio-Rad (Ivry sur Seine, France). Cy5-Tf was prepared using a labeling kit (Amersham), as indicated by the manufacturer. The antibody against rat acetylcholinesterase (Marsh et al., 1984) was kindly provided by Jean Massoulié (ENS, Paris).

Isolation and labeling of reticulocytes

Reticulocyte production in Sprague-Dawley white rats was induced by phenylhydrazine as previously described (Vidal and Stahl, 1993). After removing the buffy coat, the red blood cells (reticulocyte percentage generally >70%) were washed three times with Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4). Fluorescent phospholipid analogs were inserted into the plasma membrane, as previously described (Kok et al., 1990). Briefly, appropriate amounts of lipid, stored in chloroform/methanol (2:1), were dried under nitrogen and subsequently solubilized in absolute ethanol. This ethanolic solution was injected with a Hamilton syringe into Hanks' buffer, pH 7.4 (<1% v/v) while vigorously vortexing. The mixture was then added to the cells and an incubation was carried out for 60 minutes at 4°C after which the medium was removed, followed by extensive washing of the cells with cold Hanks' buffer. In some experiments, the cells were recovered after labeling by centrifugation on a Ficoll-Paque gradient before extensive washing with Hanks' buffer.

Fluorescence video- and confocal microscopy

Cells, mounted in buffer medium, between sealed glass coverslips and microscope slides, were viewed either by video- or confocal microscopy. Videomicroscopy was performed on a Reichert Polyvar fluorescence microscope equipped with a ×100 NA 1.32 plan apochromatic oil-immersion lens and specific rhodamine and fluorescein excitation and emission filters. Fluorescence video images were taken with a SIT camera (Lhesa Electronics, Saint-Ouen L'Aumône, France). Video signals from the SIT camera were digitally processed with a frame processing board (Data Translation DT2867) controlled by a Compaq 486 DX2-66 host microcomputer. Care was taken at the beginning of each experiment to fix the dark current level of the camera at a setting similar to previous experiments. Video images were integrated for 32 successive frames to remove electronic noise and a background image (dark current) was subtracted. Final video pictures were identically and comparatively stretched over the 256 gray levels. Confocal microscopy was performed on a Leica laser scanning microscope. Simultaneous 512×512 pixel size images (with a 32 line averaging process) of NBD-, rhodamine- and Cy5-labelled compounds were recorded using the selective set of excitation and emission filters (FITC-, TRITC, Cy5-, respectively) recommended by the manufacturer. A ×100 NA 1.30 oil-immersion lens with a pinhole size set at 100 and a zoom factor of 3.0 were used.

Recycling of membrane components

The fluorescent phospholipid analog C6-NBD-SM was inserted into the plasma membrane as described above. After extensive washing,

the cells were warmed to 37°C for 15 minutes, washed in ice-cold Hanks' buffer and back-exchanged in 5% (w/v) fat free BSA in Hanks' (HBSA) (5× 5 minutes changes) to remove surface-associated C6-NBD-lipid. After back-exchange, the reticulocytes were further incubated at 37°C for the indicated periods in HBSA, pelleted and washed once with Hanks' buffer. To quantify the amount of membrane-inserted lipid, the cells were extracted by the procedure of Folch et al. (1959). Lipid extracts were solubilized in 0.1% (v/v) Triton X-100, and fluorescence was measured using an SLM Aminco Bowman Series 2 luminescence spectrometer at 470 nm and 530 nm excitation and emission wavelengths, respectively. The fluorescence after addition of sodium dithionite (10 mM final) was taken as the background level. Sodium dithionite is a strong oxidizer which abolishes NBD fluorescence.

To monitor the intracellular flow of the Tf/TfR complex, experiments were carried out as follows. Reticulocytes (1 ml packed cells) were incubated at 4°C for 30 minutes with 0.2 ml ^{125}I-Tf (0.1 mg/ml; 4,000 cpm/ng) and warmed at 37°C for 15 minutes. Cells were extensively washed with ice-cold Hanks' buffer, followed by a brief wash with an acid solution that releases surface-bound transferrin (Sainte-Marie et al., 1991). Reticulocytes were then aliquoted and the incubation at 37°C was continued for the indicated periods with Hanks' buffer containing unlabeled Tf (0.5 mg/ml). After rapid cooling with 10 volumes ice-cold TBS, cells were pelleted, washed once with TBS, and the cell pellets were cut and counted for radioactivity using a γ-counter (Packard Cobra). In some experiments, supernatants were assessed for ^{125}I radioactivity, and confirmed the results obtained when determining the remaining cell-associated radioactivity (not shown).

Reticulocyte maturation and exosome isolation

Labeled cells were cultured (3%) for 24 hours at 37°C in maturation medium: RPMI 1640 medium supplemented with glutamine (5 mM), adenosine (5 mM), inosine (10 mM) and fetal calf serum (3%). After pelleting the cells, the culture supernatant was centrifuged (20,000 ***g*** for 20 minutes) to remove cellular debris and mitochondria. Exosomes were separated from the supernatant by centrifugation (100,000 ***g*** for 90 minutes). The pellet (vesicular fraction) was resuspended in homogenization buffer (HB: 250 mM sucrose, 1 mM EGTA, 80 mM Hepes, pH 7.4).

Analysis of exosomes

Sucrose gradient analysis

A discontinuous sucrose density gradient was prepared by sequential layering of 50, 40, 30, 20% sucrose, 1 ml each, and 0.5 ml of 10% sucrose (w/w). The samples (0.2 ml) were layered on top of the gradient. After centrifugation at 35,000 rpm for 1 hour at 4°C in an SW 50 Beckman rotor, fractions of 420 μl were collected from the bottom of the tube. Fractions obtained were analyzed (i) for N-Rh-PE fluorescence (λex = 560, λem = 590) after addition of 1.5 ml TBS ± Triton X-100 (0.1% final), and (ii) for the presence of TfR by immunoblot after blotting the fractions using a Bio-Rad dot-blot apparatus. Quantitation of the presence of the TfR was carried out on a Compaq 486 DX2-66 after digitalizing (Ikegami CCD camera) the dot blots, using an image processing and analysis home made software. The density of the fractions was determined by refractometry.

Fluorescence self-quenching measurements

Fluorescence self-quenching of N-Rh-PE was determined by measuring the fluorescence before (F0) and after (F∞) the addition of Triton X-100, using the expression (1-F0/F∞) ×100%, to calculate the percentage of self-quenching.

Exosome immunoisolation

Magnetic beads with covalently bound anti-mouse IgG (Dynal, Oslo, Norway) were incubated with a mouse anti-Tf-receptor antibody as described by the manufacturer. Exosomes were incubated for 2 hours at RT with these activated magnetic beads. The beads were then separated magnetically and washed 3 times with HBSA. Triton X-100 (0.1%) was added to release the lipids, and the supernatant was measured for rhodamine fluorescence. The non-specific isolation was quantified using an irrelevant mouse IgG instead of the anti-Tf receptor antibody.

Miscellaneous procedures

Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Phospholipid phosphorus was determined according to the method of Bartlett (1959). Radiolabeled Tf was prepared as previously described (Vidal et al., 1989). Specificity of Cy-5 binding to reticulocyte TfR was assessed by competition experiments carried out at 4°C, using excess unlabeled Tf. Immunoblotting was performed using alkaline phosphatase-conjugated antibody which was revealed by the BCIP-NBT method or using peroxidase-conjugated antibody and the ECL (Amersham) procedure. AChE activity was assayed according to the method of Ellman et al. (1961).

Fluorescent lipids were analyzed by high-performance thin layer chromatography (HPTLC) on silica gel 60 HPTLC plates (Merck). After the incubation times as indicated, the lipids were extracted from the cells and/or back-exchange medium by the procedure of Bligh and Dyer (1959). For TLC, $CHCl_3$/CH_3OH/20% (w/v) NH_4OH (14:6:1) was used as a running solvent for both fluorescent analogs.

RESULTS

Distinct trafficking of N-Rh-PE and C6-NBD-SM in reticulocytes

In reticulocytes, both analogs C6-NBD-SM and N-Rh-PE, after their initial insertion in the plasma membrane at 4°C, follow different intracellular pathways, when the cells are subsequently warmed at 37°C. As shown in Fig. 2, N-Rh-PE was rapidly internalized and after a 15 minute incubation period, punctate fluorescence was seen inside the cell (Fig. 2C). Both the number and intensity of the intracellular fluorescent dots increased with time (arrows), leading to a quasi disappearance of plasma membrane-associated fluorescence (arrowhead)(Fig. 2G). By contrast, the cells that had been labeled with C6-NBD-SM showed an entirely different labeling pattern after an incubation at 37°C (Fig. 3). Even after 4 hours at 37°C, accumulation of intracellular fluorescence was limited, while simultaneously, bright plasma membrane fluorescence was still prominently present (Fig. 3B). This plasma membrane localization was confirmed by subsequently incubating the cells in the presence of BSA (Fig. 3C). The fluorescent lipid analog present at the cell surface was removed by back-exchange, which resulted in cells displaying faintly distinguishable, intracellular fluorescence only. Finally, after the incubations, cells and back-exchanged fractions were extracted and the lipids were analyzed by HPTLC, as described in Materials and Methods. No metabolic products of both fluorescent lipid analogs were detected, neither cell-associated nor present in the media, implying that the fate of the intact N-Rh-PE and C6-NBD-SM was monitored (not shown).

To obtain a more accurate view of the trafficking of both fluorescent lipid analogs, in particular concerning their initial pathway of internalization, colocalization studies were carried out, using confocal microscopy. It should be noted that, when the plasma membrane was double-labeled with C6-NBD-SM

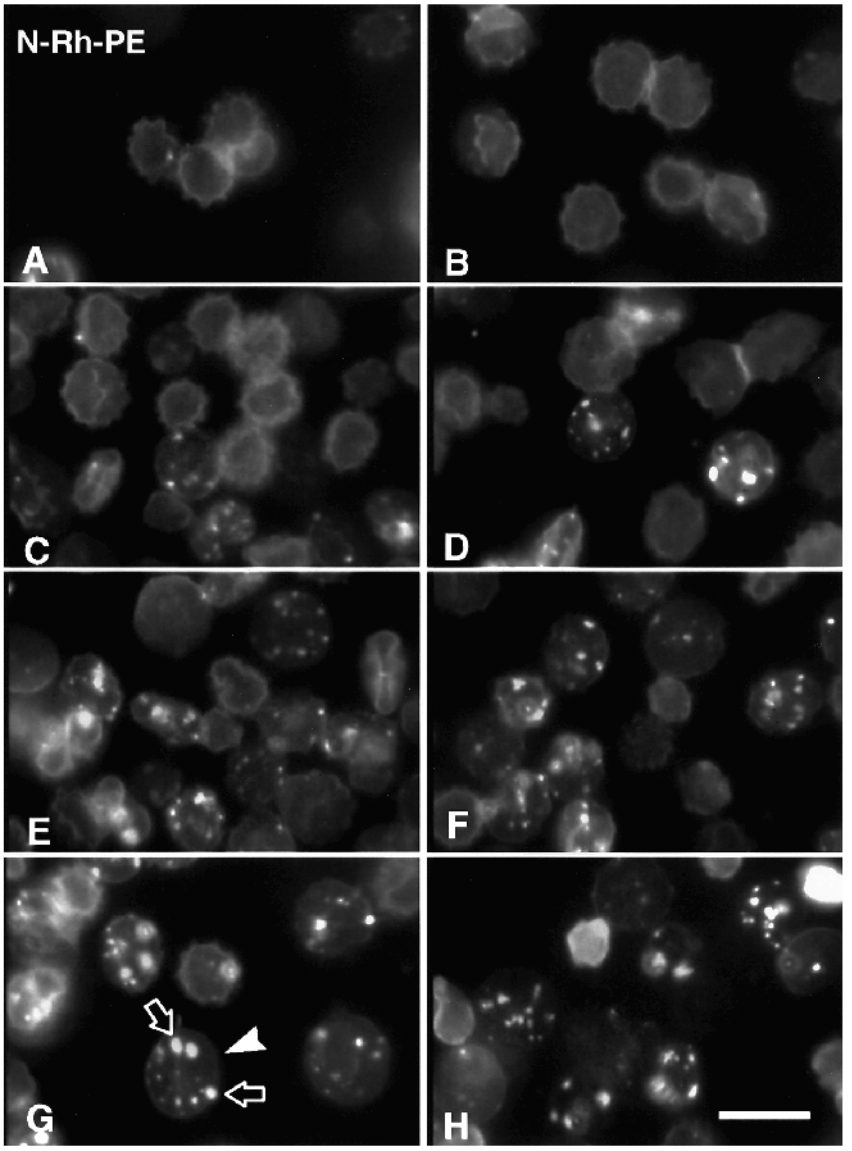

Fig. 2. N-Rh-PE internalization in reticulocytes. Reticulocytes (1 ml packed cells) were labeled at 4°C with 3 μM N-Rh-PE and extensively washed, as described in Materials and Methods. Cells were then observed by fluorescence microscopy before (A) and after incubation at 37°C for 5 minutes (B), 15 minutes (C), 30 minutes (D), 1 hour (E), 2 hours (F), 4 hours (G) and 20 hours (H). Note that the cells showing a high accumulation of internal fluorescence (arrows) display a low plasma membrane labeling (arrowhead). Bar, 10 μm.

and N-Rh-PE, a clear orange-yellow membrane staining was obtained. After 45 minutes at 37°C (Fig. 4A), the intracellular pool of C6-NBD-SM appeared marginal compared to the plasma membrane pool, and was confined to small dots. By contrast, after the same incubation period almost the entire pool of N-Rh-PE was intracellularly located (Fig. 4B). This predominant, intracellular localization was further supported by the notion, that the plasma membrane of the double labeled cells displayed bright green, i.e. C6-NBD-SM-derived fluorescence (Fig. 4C). N-Rh-PE (Fig. 4B) was associated with small vesicles and occasionally with larger structures, presumably representing MVE (cf. Fig. 2). When merging (Fig. 4C), N-Rh-PE and C6-NBD-SM were colocalized in the smaller vesicles, as evidenced by the orange-yellow appearance. The larger vesicles stained primarily red, i.e. for N-Rh-PE-derived fluorescence.

Hence, these results were consistent with a trafficking path of the lipid analogs, involving a common, endocytic internalization step, from which C6-NBD-SM subsequently flowed along the recycling pathway, while N-Rh-PE segregated into the exosomal processing pathway (cf. Fig. 1). To obtain further experimental support for these notions, the following experiments, described in the next two sections, were carried out.

The kinetics of C6-NBD-SM recycling are superimposable to those of TfR recycling

Using the capacity of BSA to efficiently back-exchange the short-chain fluorescent phospholipid analog (Fig. 3B,C), the recycling properties of C6-NBD-SM were determined (see Materials and Methods). After labeling, the cells were chased at 37°C for various time intervals in the presence of BSA, and the remaining pool of cell-associated lipid was quantified. For comparison, the recycling kinetics of preinternalized ^{125}I-Tf was also determined (Fig. 5). Both the lipid and the protein were recycled according to the same kinetics and with an equal efficiency. The half-time for recycling was about 2 minutes, consistent with previously published data (Johnstone, 1989). Direct support for this conclusion was obtained by carrying out colocalization experiments by confocal microscopy, using C6-NBD-SM and Cy5-Tf (Fig. 6). In the merged picture (Fig. 6B),

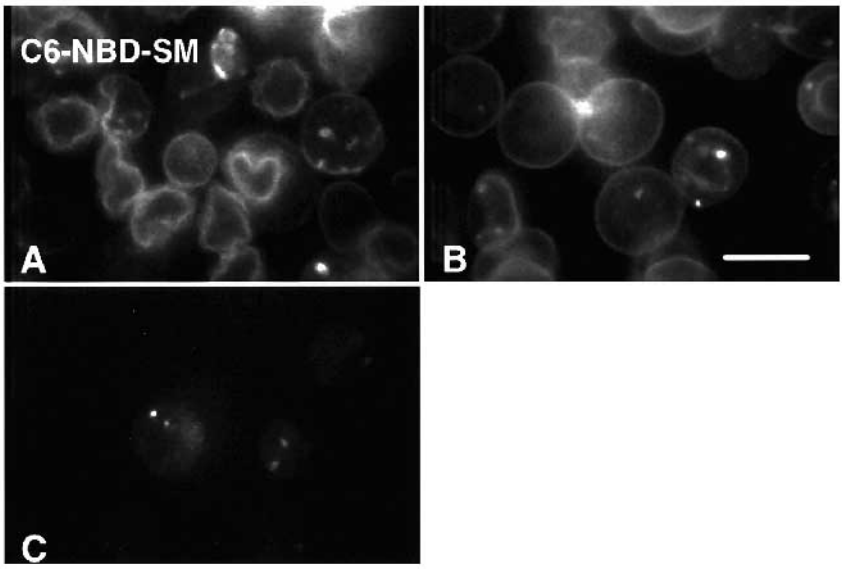

Fig. 3. C6-NBD-SM trafficking in reticulocytes. Reticulocytes (0.5 ml packed cells) were labeled at 4°C with 15 μM C6-NBD-SM and extensively washed, as described in Materials and Methods. The cells were photographed after an incubation at 37°C for 5 minutes (A) and 4 hours (B). The cells (B) were further incubated in back-exchange medium at 4°C for 15 minutes to remove fluorescent lipid located in the plasma membrane (C). Bar, 10 μm.

the extent of colocalization of Cy5-Tf with the fluorescent lipid analog was particularly visible (arrowheads). Hence, it was concluded that both SM and the Tf/TfR complex are recycled along the same direct pathway.

N-Rh-PE is associated with exosome release during the maturation of reticulocytes

The appearance of N-Rh-PE, as bright fluorescent intracellular spots in reticulocytes (Figs 2G and 4B), suggested that N-Rh-PE accumulated in the multivesicular compartment (Fig. 1), thus being sorted from the recycling pathway along which the apoTf/TfR complex, and C6-NBD-SM were primarily processed. To obtain further support for such a fate, we examined, by confocal microscopy (i) the extent of colocalization of Cy5-Tf and N-Rh-PE during their intracellular trafficking, and (ii) the flow of N-Rh-PE during reticulocyte subculturing at maturation conditions, when the Tf/TfR complex becomes sorted in the exosomal pathway (Fig. 7). After a 1 hour incubation at 37°C, the N-Rh-PE distribution showed a punctate pattern, the fluorescence being largely associated with relatively small vesicles, while occasionally larger labeled structures appeared (Fig. 7A), similar to that in Fig. 4B. Note that compared to Fig. 4, the plasma membrane was more prominently labeled with N-Rh-PE, which resulted from the higher concentration of labeling used. The distribution of Cy5-labeled Tf, after the same incubation period, displayed a similar punctate labeling pattern. Although a frequent colocalization was observed, no complete overlap occurred between both fluorescently-tagged compounds (Fig. 7C). When the cells were incubated for longer incubation periods (4 hours), N-Rh-PE stained larger vesicles (cf. Fig. 7D vs A). Interestingly, under these conditions, when significant formation of MVE occurred, Tf was detectable in all compartments that were labeled with N-Rh-PE. However, in addition, Cy5-Tf was also detected in vesicular structures that did not stain for N-Rh-PE (arrows). This distribution pattern reflected a process in which Tf recycling was still occurring, while N-Rh-PE and a gradually increasing pool of Tf were processed along the MVE pathway.

To gain biochemical support for an MVE-directed flow of N-Rh-PE during maturation, reticulocytes were labeled with the lipid analog at 4°C, washed extensively and subcultured at maturation conditions as previously described (Vidal et al., 1989). Under these conditions, the amount of cell-associated N-Rh-PE decreased with increasing maturation time. About 50% and 30% of t0 fluorescence, were still found with cells after 24 hours and 48 hours maturation, respectively. This is the time-scale of exosomal processing. Indeed, in contrast to the very rapid recycling kinetics (Fig. 5), the shedding pathway is relatively slow. This was assessed either by determining the kinetics of the decrease of the surface bound Tf fraction or by measuring by western blotting the amount of exosome-mediated release of the TfR into the extracellular medium (Fig. 8).

In parallel to a decrease in cell-associated fluorescence, an increase of N-Rh-PE fluorescence associated with exosomes was found, as determined after their collection from the medium (see Materials and Methods). Moreover, it was controlled that the recovered fluorescence had not been metabolized (not shown).

N-Rh-PE was copelleted upon isolation of TfR containing exosomes by immunoisolation (not shown, cf. Fig. 9). Furthermore, the protein composition of the exosomal fraction, as analyzed by SDS-PAGE, was found to be indistinguishable from that described previously in non-fluorescent preparations (Vidal and Stahl, 1993). In conjunction with the fluorescence microscopic observations (Figs 2,4,7), these data supported the view that during reticulocyte maturation N-Rh-PE, initially inserted into the plasma membrane, was internalized, sorted and accumulated in MVE. Subsequently, the probe was released via the expulsion of exosomes, which also contained the TfR, destined for secretion. It should be emphasized that, compared to N-Rh-PE, C6-NBD-SM was never found in significant amounts (5-10% of the total cell-associated fluorescence after 48 hours) in exosomal fractions. Consistent with this notion is the previous observation (Vidal et al., 1989) that after 48 hours of maturation approximately 8% of the total phospholipid pool of reticulocytes is released by exosomal processing. Evidently, C6-NBD-SM randomly partitions in the pool of released lipid, constituting the boundary of the

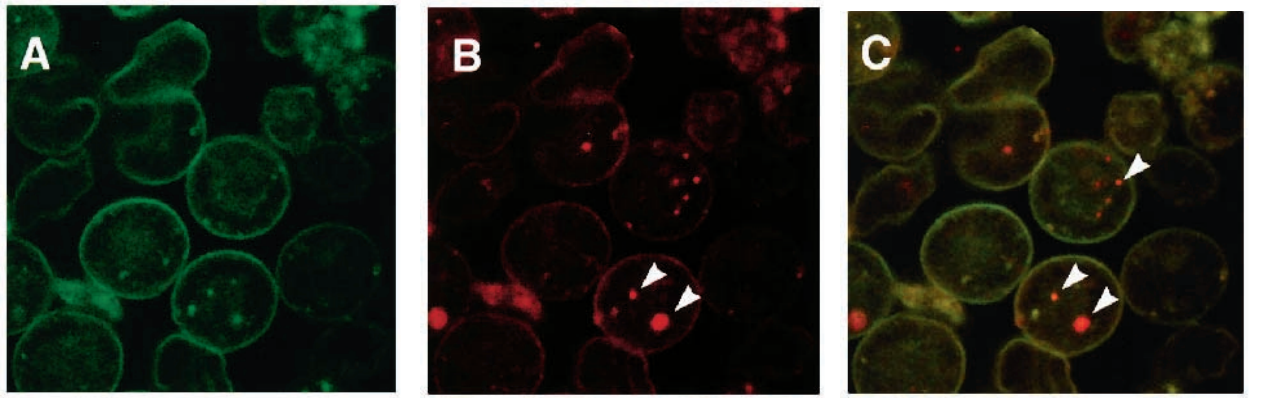

Fig. 4. Co-internalization of N-Rh-PE and C6-NBD-SM. Reticulocytes (1 ml packed cells) were labeled at 4°C with 1 μM N-Rh-PE/15μM C6-NBD-SM, and extensively washed, as described in Materials and Methods. After an incubation for 45 minutes at 37°C, the cells were examined by confocal microscopy, using appropriate filters for visualizing C6-NBD-SM (A) and N-Rh-PE (B). The merged picture is presented in C. Note that colocalization of both markers (arrowheads) causes an orange-yellow staining. 512×512 pixel size images correspond to 33×33 μm.

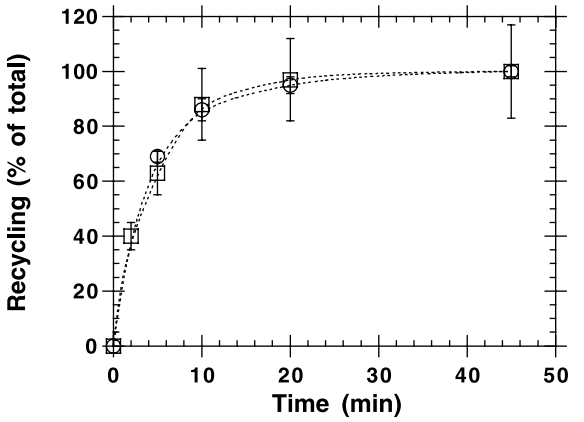

Fig. 5. Recycling kinetics of internalized C6-NBD-SM and Tf. Reticulocytes were labeled with C6-NBD-SM (90 minutes at 4°C, followed by 15 minutes at 37°C) and treated with back-exchange medium to remove C6-NBD-SM remaining at the plasma membrane. Cells were then resuspended in back-exchange medium, incubated for the indicated periods at 37°C, pelleted and extracted by the Folch procedure. The cell-associated fluorescence was quantified by spectrofluorimetry. Data (mean ± s.d.) are from typical experiments (in triplicate). Data are expressed as the recycling pool (○) from the total internal lipid fraction present at time zero, and compared to the kinetics of recycling of Tf (□) determined as described in Materials and Methods.

exosomal compartment. By contrast, N-Rh-PE is specifically processed (70% of the total cell-associated fluorescence, present at t=0, over a similar interval of 48 hours, see above), indicating a clear distinction in the (long-term) processing of N-Rh-PE and the apoTf/TfR complex on the one hand, and C6-NBD-SM on the other.

To distinguish exosomal processing from alternative mechanisms of secretion, or direct vesicle budding from the plasma membrane, the sorting mechanism was further investigated. Insight into this mechanism should also provide a molecular clue as to its ability to dictate the specific exosomal processing of N-Rh-PE and the developmentally regulated expulsion of the Tf/TfR complex.

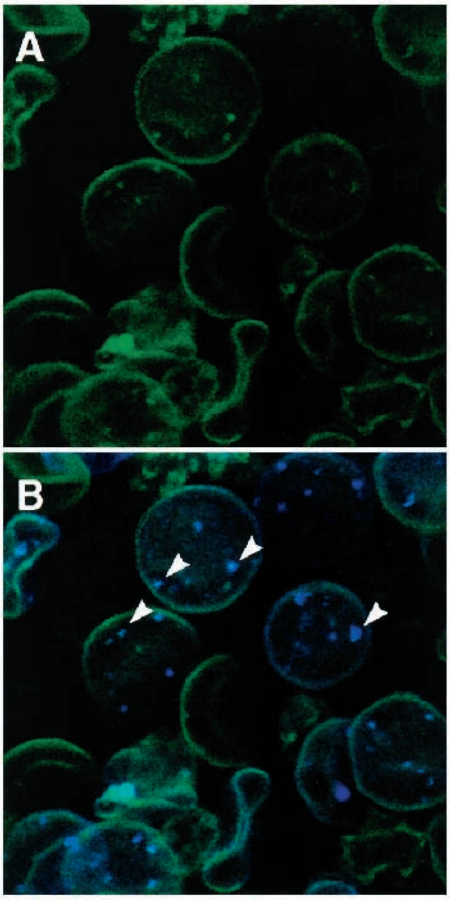

Fig. 6. Colocalization of C6-NBD-SM and Cy5-Tf. Reticulocytes were labeled with C6-NBD-SM as described in Fig. 3 and incubated for 1h at 37°C. The cells were then pulsed for 15 minutes at 37°C in the presence of Cy5-Tf (100 μg/ml final), cooled to 4°C and washed once with ice-cold Hanks' buffer. The cells were examined by confocal microscope. A typical micrograph of C6-NBD-SM localization (A) and the merged picture for C6-NBD-SM / Cy5-Tf (B) are presented. Note that colocalization of both markers (arrowheads) causes a bright blue labeling (B). 512×512 pixel size images correspond to 33×33 μm.

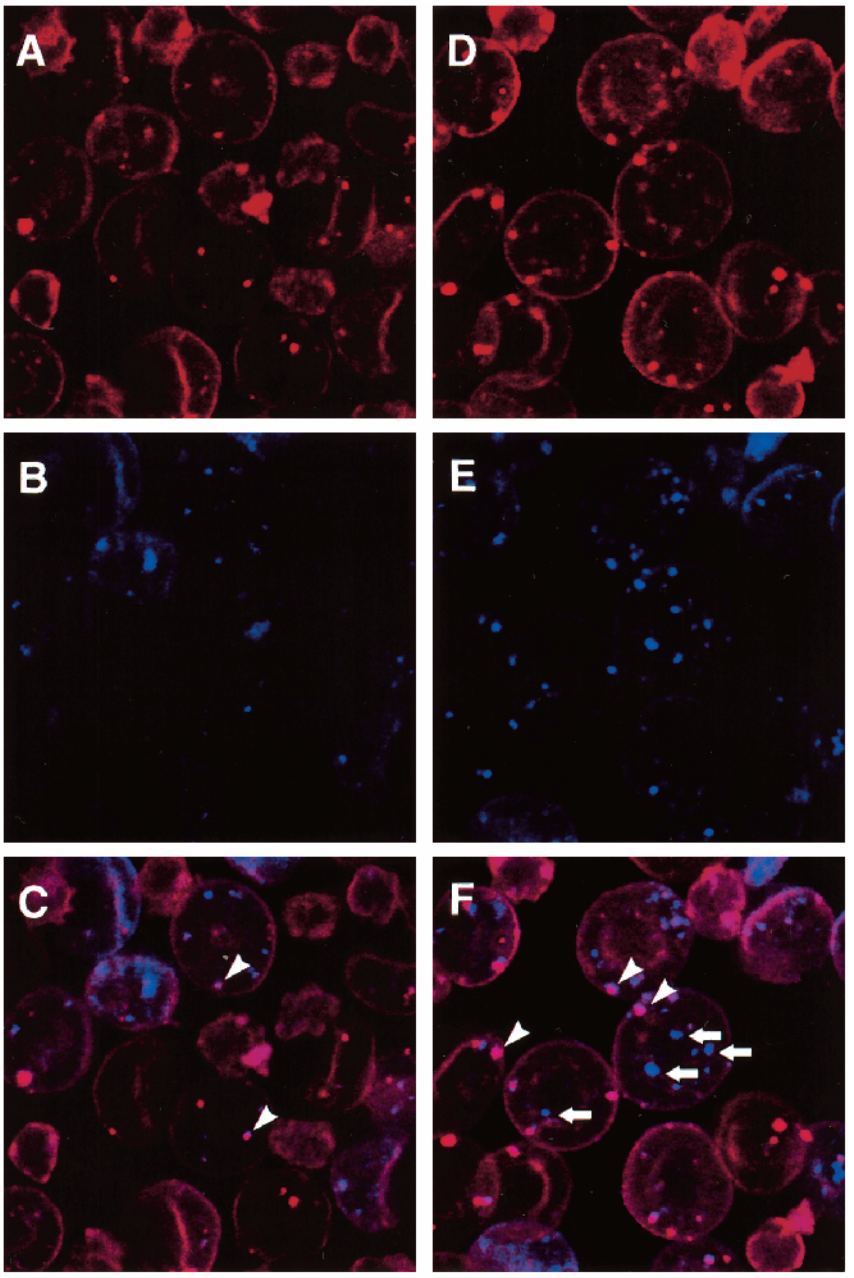

Fig. 7. Intracellular processing of N-Rh-PE and Cy5-Tf. Reticulocytes were labeled with N-Rh-PE as described in Fig. 2 and incubated at 37°C in the presence of Cy5-Tf (100 μg/ml final) for 1 hour (A-C) or 4 hours (D-F). The cells were then cooled to 4°C, washed once with ice-cold Hanks' buffer and observed by confocal microscopy for N-Rh-PE (A,D) and Cy5-Tf (B,E) localization. The merged pictures are presented in C and F. Colocalization of both markers (arrowheads) results in purple staining. Note that Cy5-Tf is detected in vesicles that are not labeled with N-Rh-PE (arrows). 512×512 pixel size images correspond to 33×33 μm.

Clustering of N-Rh-PE molecules as a potential mechanism of sorting

As described in previous work (Kok et al., 1990), exogenous insertion of N-Rh-PE into the plasma membrane presumably results in the lipid's intercalation in the membrane as small clusters of lipid molecules. Nevertheless, values for the lateral diffusion rate constant and mobile fraction are very similar to values obtained for lipid analogs, known to be inserted as monomers. Yet, clustering is revealed by observations of fluorescence self-quenching, the extent of which is much higher than would be anticipated, based upon the concentration-dependent increase in fluorescence self-quenching. Consequently, it has been hypothesized (Kok et al., 1990), that small molecular aggregates may trigger a mechanism that causes N-Rh-PE patches to be specifically removed from the cell surface by endocytosis, resulting in lysosomal delivery. It was possible that such a mechanism was also operative in the sorting and expulsion of N-Rh-PE in reticulocytes, lysosomal delivery being analogous to exosomal expulsion. To test this hypothesis, experiments were designed to diminish or eliminate N-Rh-PE patch formation by 'dilution' of the analog with DOPE, prior to membrane insertion via ethanol injection. It was anticipated that such a 'dilution' should reduce inter-molecular N-Rh-PE interactions, thus enhancing the proportion of the lipid-analog being processed as a 'monomer' rather than a molecular 'cluster'.

When an ethanolic solution of pure N-Rh-PE was injected into the buffer, a maximal quenching of Rh fluorescence was obtained (approx. 95%) as revealed by measuring fluorescence before and after addition of Triton X-100, respectively (Table 1). The same efficiency of quenching was determined for the plasma membrane-associated fluorescence and that in

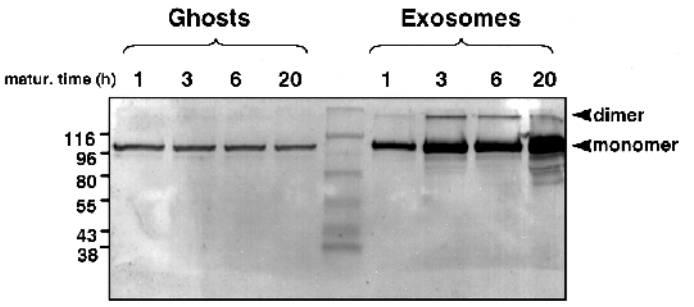

Fig. 8. TfR release during reticulocyte maturation. 0.3 ml packed reticulocytes were subcultured in 10 ml maturation medium for the indicated periods at 37°C. Exosomes were then collected from the culture medium as described in Materials and Methods. The plasma membrane fraction ('ghosts') from corresponding remnant cells was prepared as previously described (Vidal and Stahl, 1993). Samples (i.e. the total exosome fractions and aliquots (1/50) of the plasma membrane fractions) were subjected to SDS-PAGE in a 10% acrylamide gel and proteins were transferred to nitrocellulose. TfR was detected by western blot using a monoclonal antibody raised against rat TfR and an alkaline phosphatase-conjugated rabbit anti-mouse antibody. The molecular mass (kDa) standards are indicated to the left.

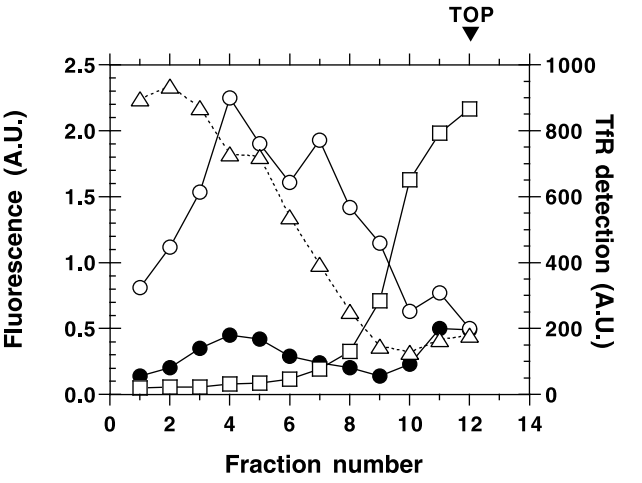

Fig. 9. Sucrose gradient analysis of N-Rh-PE labeled exosomes. Exosomes obtained from reticulocytes, labeled with N-Rh-PE (4 mol % in DOPE ethanolic micelles, see text) were analyzed by sucrose gradient centrifugation as described in Materials and Methods. Fractions collected were measured for fluorescence before (●) and after (○) addition of detergent. Samples were blotted on nitrocellulose and analyzed for the presence of TfR by western blot. Quantitation of the amount of TfR determined after scanning the western blot is plotted (△). A sample of buffer containing N-Rh-PE/DOPE micelles was identically analyzed by gradient centrifugation, and fluorescence present in each fraction was measured after addition of detergent (□).

exosomes, isolated from the N-Rh-PE labeled cells. The fluorescence self-quenching decreased when mixed micelles, consisting of N-Rh-PE and DOPE, were added to the cells, consistent with the anticipated molecular dilution of N-Rh-PE. As shown in Table 1, exosomes isolated from matured reticulocytes that had been labeled with such relatively low Rh-PE:DOPE ratios, showed a persistently higher degree of quenching than that in the plasma membrane. These data were consistent with the notion that after internalization, N-Rh-PE was sorted from other lipids and packaged in the exosomal membrane. Indeed, such exosomes could be specifically isolated by sucrose gradient fractionation.

As shown in Fig. 9, when fractionated on a sucrose gradient, N-Rh-PE containing micelles were recovered, floating on top of the gradient. A different distribution pattern of fluorescence was obtained when exosomes, shed by reticulocytes after labeling with a Rh-PE:DOPE ratio (4%) were similarly analyzed. Both fluorescence and quenching were maximal in fraction 4, i.e. a fraction of much higher density than the fraction in which the micelles per se were recovered (1.15 vs 1.08 g/ml). Moreover, it should be noted that no significant fluorescence quenching was detected in the top fractions (11 and 12). That result was consistent with an efficient membrane lipid insertion (see also Kok et al., 1990) and subsequent processing, and excluded an artificial adsorption of micelles. Interestingly, and entirely consistent with the microscopy and immunological data described above, there was a substantial co-recovery of the fluorescent lipid analog with TfR-containing fractions. Hence, these data strongly argued in favor of a sorting and concentration step of N-Rh-PE into exosomes during cell maturation.

From these results, the question then arose whether the aggregation per se led to sorting and packaging into exosomal structures, or that proteins, accidentally colocalizing in N-Rh-PE-enriched regions, would accompany lipid patches. It was therefore of particular relevance to determine whether aggregation of proteins, including the TfR, could lead to exosomal processing.

Table 1. Sorting of N-Rh-PE and exosomal processing

	N-Rh-PE:DOPE ratio (%)			
	0.4	1	4	100
Buffer	0	8.5±3.5	60±1.7	94±3.6
Cells	0	0	56±3.6	90±4.2
Exosomes	18.7±7.4	38.5±3.5	60±4.1	75±6.1

Ethanolic solutions of pure N-Rh-PE or N-Rh-PE/DOPE mixtures were injected into Hanks' buffer. The resulting fluorescence (self)quenching was determined by measuring aliquots of the labeled buffers (Buffer), as described in Materials and Methods. Reticulocytes were labeled at 4°C with these buffers and washed. Subsequently, the extent of (self-)quenching of cell-associated fluorescence was determined (Cells). The incubation was then continued in maturation medium and exosomes were collected as described in Materials and Methods. Fluorescence self-quenching was determined in this vesicular fraction (Exosomes). Data presented are mean ± s.d. of at least 3 experiments.

Protein sorting in exosomes is enhanced by aggregation

Both Tf and TfR contain mannose residues which interact with the plant lectin concanavalin A (ConA). As shown in Fig. 10A, the presence of ConA during reticulocyte maturation caused an enhancement of the amount of TfR secreted via exosomes. By contrast, when adding the lectin at the end of the maturation period, no effect on the TfR release was observed (data not shown). This control experiment also ruled out the possibility that the lectin favored pelleting of exosomes, as a result of lectin-mediated cross-linking.

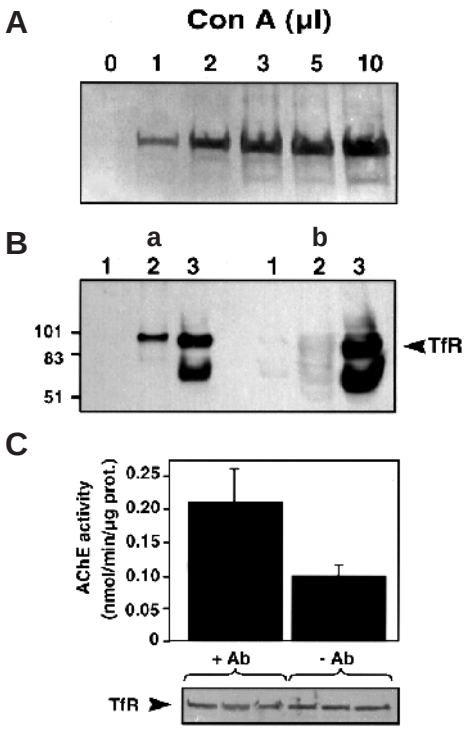

Fig. 10. Protein sorting in exosomes is enhanced by aggregation. (A) Effect of ConA on TfR release during maturation. Reticulocytes (0.3 ml packed cells) were incubated in 10 ml maturation medium. After preincubation (30 minutes at 37°C) with 250 μg transferrin, indicated volumes of concanavalin A (16 mg/ml) were added during reticulocyte maturation. Exosomes were then collected from the medium and analyzed for the presence of TfR by western blot as in Fig. 8. (B) TfR release with exosomes is enhanced by specific cross-linking. (a) Reticulocytes (0.3 ml packed cells) were incubated in 10 ml maturation medium (lane 1) supplemented with 10 μl anti-TfR antibody (lanes 2, 3). After 30 minutes at 37°C, 100 μl rabbit anti-mouse IgG antibody were added to the cells (lane 3). Cell maturation was carried out for 24 hours. Exosomes were then collected from the maturation medium and analyzed by western blotting the TfR using the enhanced chemiluminescence method. (b) Exosomes were collected and analyzed as in (a), after reticulocyte maturation in the presence of 160 μg/ml Tf (lanes 1-3), supplemented with 100 μl goat anti-Tf (lanes 2,3) and rabbit anti-goat IgG (lane 3) antibodies. The molecular mass (kDa) standards are indicated to the left. The lower band (approx. 70 kDa) is the truncated form of the TfR (Johnstone, 1996). (C) Antibody-mediated cross-linking of AChE favors its exosomal release. Reticulocytes (0.3 ml packed cells) were incubated in 10 ml maturation medium supplemented with 3 μl anti-AChE antibody. After 30 minutes at 37°C, 30 μl sheep anti-rabbit IgG antibody were added to the cells (+Ab). Cell maturation was carried out for 24 hours in triplicate. Control maturation was done in the absence of antibodies (–Ab). Exosomes were then collected from the maturation medium and analyzed for AChE activity (mean ± s.d.) as described in Materials and Methods, and by western blotting the TfR as in Fig.8.

To specifically cross-link Tf receptors, reticulocytes were incubated in the presence of antibodies directed against TfR or against Tf (Fig. 10B). The indirect cross-linking involved the addition of the specific ligand (i.e. transferrin) previously incubated with anti-Tf antibody. Both direct and indirect cross-linking of TfR resulted in a dramatic enhancement of the amount of TfR released within exosomes. It is especially remarkable that the addition of the second stage antibodies during the maturation led to the highest amounts of TfR released (lanes 3 vs lanes 1,2), suggesting that the larger were the TfR clusters, the more efficient became their rerouting into exosomes.

AChE is another membrane protein, which is specifically released in exosomes during reticulocyte maturation (Johnstone et al., 1987). Using the same approach, AChE was cross-linked at the cell surface before incubation at maturation conditions. As shown in Fig. 10C, cross-linking of the enzyme more than doubled the increase of the AChE activity detected in the released exosomal vesicles, whereas the amount of TfR released under these conditions was unaltered, implying that only clustering facilitated shedding.

DISCUSSION

Membrane traffic during reticulocyte maturation

Because organelles like Golgi and endoplasmic reticulum are virtually absent in reticulocytes, these cells represent a unique system to investigate distinct aspects of intracellular trafficking occurring in the endocytic pathway. Membrane trafficking in this pathway is essentially limited to the internalization and/or recycling of a distinct set of proteins, including the Tf/TfR complex. Eventually these proteins are eliminated by expulsion via vesicular carriers, called exosomes (Fig. 1). In the present work we have shown that the flow and fate of the endocytic vesicles can be conveniently monitored using fluorescent phospholipid analogs, and that depending of the nature of the analog, either efficient recycling of a lipid occurs, with kinetics identical to those of the TfR complex, or elimination through exosomal release takes place. Molecular clustering of either proteins or lipids favors exosomal processing.

Recycling versus sorting

Both the TfR and the fluorescently-tagged SM are internalized and similarly rapidly recycled in developing reticulocytes ($t1/2$ of approx. 2 minutes). Moreover, C6-NBD-SM and Tf colocalize during the early phase of the internalization process. Thus, given the limited membrane flow pathways in these cells, it is reasonable to conclude that both membrane markers follow the same trafficking route during the initial stages of reticulocyte maturation.

Over longer periods of time (hours; Fig. 8), the TfR is secreted into the extracellular milieu and the protein is recovered in exosomal vesicles. Under these conditions, TfR dimers were often detected on gels, in spite of the strong reducing conditions used (cf. also Ahn and Johnstone, 1993). This might reflect the possibility that strong intermolecular interactions exist at maturation conditions. Indeed, we document here that molecular clustering of either proteins or lipids in the membrane (‘patching’) represent an important parameter to govern the rerouting of a molecule from a recycling path to elimination. This result is consistent with previous studies of lysosomal targeting of grossly crosslinked receptors, either by large multivalent ligands (Marsh et al., 1995) or by anti-receptor antibodies (Mellman and Plutner, 1984; Weismann et al., 1986; Lesley et al., 1989). Since reticulocytes lack such a degradative compartment, an effective

alternative is provided by an analogous processing along the exosomal pathway.

Sorting and exosomal processing

Using the fluorescent lipid analog N-Rh-PE, evidence was obtained that the clustered analog was specifically processed along the exosomal pathway. Moreover, clustering involves a sorting event that occurred subsequently to the internalization of the lipid analog, within the lateral plane of the MVE, as revealed by experiment of lipid insertion from a more 'dilute' source of mixed micelles (Table 1). In addition, four pieces of evidence support the conclusion that exosomal processing indeed took place. First, fluorescence microscopy shows its association with an intracellular vacuolar system which, given its size, certainly represents MVE, the only organelle in reticulocytes (Figs 2, 4). Second, co-labeling studies, demonstrate the presence of N-Rh-PE in TfR-containing vesicles, the precursors of exosomal vesicles (Fig. 7). Third, the lipid analog is recovered in a fraction of the extracellular medium, previously identified as exosomes (Vidal and Stahl, 1993). Immunoisolation of this fraction with TfR-antibodies shows a colocalization of N-Rh-PE and TfR, implying their joint presence in a vesicular compartment. Fourth, sucrose gradient analysis of exosomes isolated after labeling the plasma membrane with a low ratio of N-Rh-PE, confirmed this colocalization (Fig. 9).

In maturing reticulocytes, i.e. at physiological conditions, a distinct set of proteins will also be gradually eliminated from the cells by sorting and processing via exosomal secretion. By analogy with the fate of clustered N-Rh-PE, this scenario is facilitated when such proteins are cross-linked, as was shown for the Tf/TfR complex and AChE, a GPI-linked membrane protein. Although a variety of sorting motifs in proteins have been demonstrated (Trowbridge, 1991; Sandoval and Bakke, 1994), signals are not strictly required in reticulocytes, given the relatively simple 'organellar' organization in these cells. Hence, 'patching' would represent the most simple and efficient option for effective processing in reticulocytes. It is very likely that in vivo clustering, simulated in the present work by use of antibodies or a lectin, involves the participation of specific triggers and/or chaperones, such as hsc70 (Johnstone, 1992). Nevertheless, a driving force would be required for directing the aggregate-mediated formation and packaging into exosomes, the mechanism of which is also as yet entirely unclear. In this context, it is likely that cross-linking acts as a lumenal retention signal in a recycling compartment (cf. Marsh et al., 1995), i.e. MVE in case of reticulocytes. Since the inward rate of membrane flow by endocytosis would then exceed the outward flow by recycling, exosomal vesicle formation can be readily rationalized. However, it is at present unclear whether endocytic vesicles (i) interact with MVE at early stages of maturation (see Fig. 1; Vidal and Hoekstra, 1995), (ii) directly 'mature' as new MVE, or (iii) are only targeted to the MVE compartment when they contain clustered 'cargo'. Since vesiculation may be triggered by compounds that drive a process which generates domains of high curvature, patching itself could trigger vesiculation of the endosomal membrane. Such effects have been noted in liposomes composed of PE and GD1a (van Gorkom et al., 1995).

Clustering or patching, preceding vesiculation would not be without precedent. Glycosphingolipids tend to form clusters within bilayers (Thompson and Thillack, 1985), possibly mediated by intermolecular hydrogen bonding. Formation of such domains, which may include specifically clustered proteins as well, is thought to act as a sorting event for a 'programmed' delivery to distinct membrane domains in polarized cells (Simons and Wandinger-Ness, 1990; Glaser, 1993). Similarly, such a sorting step in an endosomal compartment could provide a molecular mechanism, causing elimination of obsolescent molecules either by lysosomal digestion or exosomal secretion. The former has been noted for N-Rh-PE molecules, processed as such in baby hamster kidney cells (Kok et al., 1990). Distinct glycosphingolipids have been proposed to be processed according to such a mechanism (Sandhoff and Klein, 1994), the similarity being that both types of molecules show an inherent tendency to self-aggregate. The effectiveness of N-Rh-PE packaging via vesiculation would agree with observations that the extent of MVB formation in the endocytic pathway is influenced by the fate of the molecule involved. Thus recycling receptors generate relatively few internal vesicles, whereas substantial vesiculation occurs upon processing of proteins (ligands and/or receptors) destined for degradation (Futter et al., 1993; van Deurs et al., 1993).

It is finally of interest to note that, given that N-Rh-PE does not cross the membrane, the signal for vesiculation after patching apparently does not require a transmembrane entity. Indeed, it has been shown that truncated TfR, lacking the extracellular domain, which faces the endosomal lumen when inserted in the endosomal membrane, is still sorted in exosomes upon maturation (Ahn and Johnstone, 1993). This suggests that the cytosolic domain of the TfR is sufficient for triggering the receptor to exosomes. A partial denaturation of the cytosolic domain of the TfR as a cause that leads to MVE-mediated elimination has been proposed (Johnstone, 1992), and could thus trigger clustering at physiological conditions. On the other hand, although cytosolic parameters might be involved, it appears no prerequisite, since N-Rh-PE does not span the bilayer but, rather, consistently faces the lumenal site during its intracellular flow in reticulocytes. In accordance with this, acetylcholinesterase has been shown to be released in exosomes with a yield proportional to its decrease (around 50%) from the cell surface during red cell maturation (Johnstone et al., 1987). In mammalian erythrocytes (Toutant et al., 1989) and in rat exosomes (J. P. Toutant and M. Vidal, unpublished observations), acetylcholinesterase is a GPI-anchored membrane protein, whose activity can be totally released by PI-PLC treatment. Therefore, AChE, similar to N-Rh-PE, does not cross the bilayer. This observation further emphasizes the possibility that under physiological conditions, involvement of a cytosolic signal in the sorting of AChE can be excluded. Obviously, a common sorting device, dictated by the specific exosomal processing of the clustered lipid and proteins as described in the present study, may then involve the formation of specific lipid rafts. However, such a sorting mechanism remains to be determined.

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