In Vitro Fusion of Reticulocyte Endocytic Vesicles with Liposomes*

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Since reticulocytes have a high demand for iron, which is required for heme biosynthesis, these cells are highly specialized in the endocytosis of the iron carrier transferrin (Tf). From the resulting endocytic vesicles (EVs), iron is released and the vesicles rapidly return to the cell membrane where they fuse, causing the release of the apotransferrin. Due to a lack of other intracellular compartments, the endocytic vesicles can be readily isolated. In this study, we have investigated the fusogenic properties of EVs, using liposomes as target membranes. Membrane fusion was monitored by a lipid mixing assay based on the relief of fluorescence self-quenching, using octadecylrhodamine B-chloride (R18). Application of this procedure was verified and solidified by analysis of the fusogenic event by an independent lipid mixing assay, after in situ labeling of EVs, and by determination of the mixing of aqueous contents.

We demonstrate that the endocytic vesicles are particularly prone to fuse with target membranes that contain dioleoylphosphatidylethanolamine (DOPE). Relative to DOPE, bilayers composed of phosphatidylserine or phosphatidylcholine show a reduced fusion activity with EV. The specific and strong inhibition of fusion by cyclosporin A and a peptide known to interfere with the propensity of DOPE to adopt the hexagonal II phase suggests that the mechanism of fusion involves the ability of this lipid to readily adopt non-bilayer phases. ATP, GTP, and/or cytosol are not necessary to obtain fusion. However, trypsin treatment of the endocytic vesicles inhibits fusion, indicating the involvement of (α) protein(s) in the fusion event.

Vesicular transport along the endocytic and secretory pathways involves the budding of vesicles from a donor compartment, specific targeting of the vesicles to an acceptor compartment, followed by a fusion event between target and vesicular membranes. To investigate the mechanisms and regulation of such vesicular transport processes, many cell-free systems have been developed to reconstitute intercompartmental transport. This has led to the recognition and identification of a growing number of molecular factors (proteins, nucleotides) thought to be involved in the distinct interaction steps (1, 2). With regard to budding, adaptins and clathrin have been recognized in the endocytic pathway as the major proteins involved in receptor sorting and vesicle formation from plasma membrane and trans Golgi network (3). As to the subsequent steps in intracellular trafficking, i.e., vesicle docking and fusion, a host of molecules have been identified, including small GTPases (rab proteins), heterotrimeric G proteins, NSF, SNAP, and SNARE. The function of most of these factors is largely obscure, although some of these components may well be part of a machinery, common to all vesicle fusion events, as unified in the so-called SNARE hypothesis (4). According to this hypothesis, SNAPs bind to their receptors, SNAREs, on the vesicle (v-SNARE) and on the target membrane (t-SNARE). NSF subsequently binds to SNAPs, which causes ATP hydrolysis, thus inducing complex dissolution, which may precede or follow the merging of membranes. Recently, it has been shown that celubrevin, a member of the SNARE family (5), is involved in the recycling of the Tff receptor, as observed in permeabilized Chinese hamster ovary cells (6). NSF, first described by Rothman and collaborators (7), has been shown to be crucial to homotypic fusion of endosomes (8). The functioning of rab proteins in the fusion of endocytic vesicles does not seem to depend on the GTPase activity, as revealed by the use of rab GTPase mutants (9) and fusion experiments in the presence of nonhydrolyzable GTP (10). However, it is evident that the molecular mechanism of membrane fusion is far from understood.

Receptor-mediated endocytosis refers to the cell's capacity to internalize components after their binding to specific receptors. Depending on the receptor–ligand complex involved, molecules are then delivered to the lysosomes for degradation. Reticulocytes are highly exceptional in that they are hyperspecialized for uptake of iron, which is needed for the biosynthesis of heme. In fact, to meet this requirement, endocytic membrane traffic consists primarily of the transferrin–transferrin receptor complex constituting the sole complex internalized by reticulocytes. As indicated by the quasi-absence of lysosomes, there is no sorting event in the endosomal compartment. Rather, after release of iron, Tf rapidly recycles to the cell surface (5 min; for sheep reticulocytes (11); less than 4 min for rat reticulocytes (12)), making Tf available for another round of iron transport. Apart from the few lysosomes left, reticulocytes are also anucleate cells containing only vestigial remnants of Golgi and endoplasmic reticulum (13). Hence, the virtual lack of other internal compartments and their highly specific endocytic function makes these cells an excellent source and model to isolate and study the properties, respectively, of endocytic vesicles. Previously, it has been shown that these vesicles contain several small GTPases, which presumably regulate the intracellular trafficking in the cell (14). In the present work, we have initiated a study aimed at investigating the fusogenic properties of the endocytic vesicles. To bypass molecular factors that regulate trafficking and docking, although essential prerequi-

1 The abbreviations used are: Tf, transferrin; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; EV, endocytic vesicle; N-Rh-PE, N-dissamine rhodamine B sulfonyl-1-phosphatidyethanolamine; R18, octadecylrhodamine B-chloride; Z-PG, carboxyoxo-D-phenylalanyl-L-phenylalanylglutamic acid; PC, phosphatidylcholine; PS, phosphatidylserine; MES, 4-morpholinethanesulfonic acid; GTP·S, guanosine 5'-O-(thiotriphosphate).

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sites to fusion in vivo, we have used artificial membranes as target membranes. Various assays based on lipid mixing and content mixing were employed, allowing us to conveniently determine initial kinetics of endocytic vesicle fusion. Thus parameters that affect the fusion process can be readily evaluated in this manner. We show that fusion is rapid, temperature dependent, and is markedly affected by the lipid composition of the target membrane, strongly supporting a role for lipids capable of forming hexagonal lipid phases. Fusion is mediated by a trypsin-sensitive factor, which is present on the membrane of endocytic vesicles.

EXPERIMENTAL PROCEDURES

Materials—Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), phosphatidylserine (PS, bovine brain), cholesterol, and carbethoxy-diphosphatidylglycerol (Z-FFG) were purchased from Sigma. Octadecylrhodamine B-chloride (R18), N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (N-Rh-PE) were obtained from Molecular Probes (Eugene, OR). Cyclosporin A was from Sandoz (Basel, Switzerland). Palmitoyl-L-1-

Preparation of Liposomes—Large unilamellar vesicles were prepared as described by Kremer et al. (16). Briefly, lipids were mixed from stock solutions, dried under nitrogen, and resuspended in ethanol. The ethanolic solution of phospholipid was dried from the ethanol by a stream of nitrogen. A Hamilton syringe was used to inject 2.5 ml of saline buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4) into a 5-mm Tube; 1 ml of buffer was injected, under vigorous stirring. By quasi-elastic light scattering using a Coulter particle analyzer (model N4S), it was determined that the liposome size of the various DOPC/DOPE-containing vesicles varied between 140 and 360 nm. The size of DOPC and PS vesicles varied between 110 and 130 nm. Note that this similarity in vesicle sizes will exclude a significant contribution of curvature to the fusion kinetics. Phospholipid content was quantified as described by Bardgett (16). Preparing of Endocytic Vesicles—Anemia was induced in Sprague-Dawley rats by phenylhydrazine injections. Blood was withdrawn by heart puncture and centrifuged at 1000 × g for 5 min. After removing the buffy coat, the red blood cells (reticulocyte percentage was generally >70%) were washed three times with the saline buffer. Endocytic vesicles (EVs) were prepared as described previously (14). Briefly, the cells were resuspended (50% hematocrit) in lysis buffer (50 mM NaCl, 50 mM KCl, 1 mM EDTA, 0.5 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride, 3 mM Na2CP, 100 mM MES, pH 6.5) and lysed by two cycles of rapid freezing followed by thawing with gentle agitation at 4 °C. The non-lysed cells and debris were pelleted at 20,000 × g for 20 min, and the supernatant was ultracentrifuged at 65,000 × g for 60 min. The pellet enriched with EVs was gently resuspended with 250 ml PBS containing 20 mM HEPES-KOH, pH 7.0, at a protein concentration of about 5 mg/ml, and kept in aliquots at –80 °C.

For the gradient analysis experiments, the EVs were labeled with N-Rh-PE or 125I-Ty. To this end, reticulocytes were preincubated at 4 °C for 1 h in RPMI containing N-Rh-PE (50 μM solubilized in ethanol; final ethanol concentration <1%, v/v) or 125I-Ty (20 μg/ml). To trigger membrane internalization, the cells were warmed to 37 °C. After 30 min, the cells were washed three times with saline buffer, and EVs were isolated as described above. TF-labeled EV preparations were loaded onto a Sephacryl S-200 column (20 × 1 cm) equilibrated with saline buffer or to ovalbumin free 125I-Ty were gradient fractionated.

Labeling of Endocytic Vesicles with R18—EVs were labeled with R18 by injection (under vigorous vortexing) of 10 μl of an ethanolic solution of R18 (14 μM) into 1 ml of saline buffer containing the vesicles (1 mg protein/ml, 1.3 mM phospholipid). The mixture was incubated on ice for 1 h in the dark. Labeled EVs were separated from unincorporated R18 using a Sephadex G-75 column (20 × 1 cm), equilibrated with saline buffer. By measuring R18 fluorescence versus phospholipid prior to and after gel filtration, it could be determined that the efficiency of labeling varied from 50–70%. This implies that the final EV preparations contained ~85% R18 with respect to total phospholipid, i.e., roughly 2.5–3.5 % with respect to total lipid. These numbers are similar to the extent and efficiency of labeling seen for various viruses (18). Aliquots of R18-EVs were kept at –20 °C. The probe remained firmly membrane associated and no release was seen (as determined by gel filtration) upon prolonged storage nor when the labeled membranes were incubated at 37 °C for 60 min. Neither was significant degradation of the probe apparent after a 1-h incubation at 37 °C, as revealed by lipid extraction and analysis by thin-layer chromatography. This is consistent with the notion that nonspecific esterase activity is not associated with EVs (44). Quenching (Q) was calculated according to Q = 1 – F/F0, where F is fluorescence and F0, the fluorescence measured after addition of Triton X-100 (0.5% v/v).

Immunosioisolation of R18-EVs was carried out using S. aureus, coated without an antibody raised against a peptide (24 AA) of the cytosolic domain of the TR (17), generously provided by Rockford Drake (University of Texas, Dallas, TX).

Fusion Assay—Fusion of R18-EVs with liposomes was measured by the R18 assay (18). Fluorescence was measured using a Fluoromax III spectrofluorometer attached to a 300-W xenon arc lamp. Glucose solution was added to the cuvette to bring the concentration to 150 mM. The cuvette was then sealed and fluorescence was recorded. The excitation and emission wavelengths were set at 520 nm and 540 nm, respectively. The fluorescence was obtained by injection of 200 μl of liposomes (2 mM stock) into a magnetically stirred cuvette containing the appropriate amount of R18-EVs in saline buffer. The final incubation volume was 2 ml. The residual fluorescence of R18-EVs in the cuvette, before addition of liposomes, was set to 0%. The fluorescence after addition of Triton X-100 (0.5% v/v), corrected for dilution, was taken as 100%. Data are expressed as the initial rate of fusion, determined from the fluorescence tracings at time 0.

Sucrose Gradient Analysis of Fusion Products—A discontinuous sucrose gradient was prepared by sequential layering of 50, 40, 30, and 20% sucrose, 1 ml each, and 0.5 ml of 10% sucrose (w/v). The samples (0.25 ml), containing either EVs or liposomes or both, were layered on top of the gradient. After centrifugation at 35,000 revolutions/min for 1 h at 4 °C in an SW 50 Beckman rotor, fractions of 450 μl were collected and assayed for EVs, liposomes, and EVs + liposomes. Depending on the experiment, aliquots of the fractions were (i) analyzed for N-Rh-PE fluorescence (after addition of Triton X-100 (1 mg/ml, 1 mg/ml); (ii) counted in a β-counter (Beckman LS 5800DD) to quantify the presence of [125I]CPC, or (iii) counted in a γ-counter (Packard Cobra) to determine 125I-Ty.

RESULTS

Labeling of EVs with R18—Endocytic vesicles were prepared as described under “Experimental Procedures.” The characteristics of the EV preparation were similar to those previously described (14), including the enrichment of the TR-F as assessed by Western blots. EVs thus prepared were then labeled with R18 using an ethanol injection method (18). It has been shown that this method allows the introduction of the probe into biological membranes, resulting in a concentration-dependent self-quenching of fluorescence. At the conditions described under “Experimental Procedures,” incorporation of R18 was such that the quenching of fluorescence was usually higher than 0.9.

To confirm that R18 was specifically incorporated in endocytic vesicles, we immunosioisolated the vesicles via the TR-F and analyzed the associated fluorescence. The immunosioisolation was carried out using S. aureus coated with an antibody raised against a defined peptide containing the cytosolic domain of the TR (17). S. aureus coated with the anti-TR-F was able to pellet three times more R18 fluorescence than S. aureus coated with an irrelevant antibody, thus confirming the colocalization of lipid probe and TR-F in EVs.

Lipid Mixing as a Measure of EV Fusion Activity—To determine the fusion activity of EVs, the R18-labeled vesicles were mixed with unlabeled liposomes. The protocol implies that fusion of both membranes will result in dilution of the fluorescent lipid analog, causing relief of self-quenching. Hence an increase in fluorescence reports, in principle, the occurrence of fusion. As shown in Fig. 1A (curve a), addition of the liposomes to R18-labeled EVs resulted in a time-dependent increase of fluorescence. From the slope of the fluorescence tracing at time 0, the initial rate of fluorescence increase was calculated (Fig. 1B). Typically, the rate of lipid mixing was in the order of approximately 5%/min, while the increase of fluorescence development leveled off after 15–20 min (Fig. 2). As shown in Fig. 2A, both the rate and bottom of dilution depend on the concentration of liposomes, showing an increase when the concentration increases. Moreover, when the saline buffer was replaced by an isososmotic sucrose buffer, lipid mixing was virtually
Indeed, a shift in density was observed when R18-labeled EVs had been incubated with liposomes for 30 min at 37 °C (not shown). An identical result was obtained when EVs were labeled endogenously. This was accomplished by labeling the reticuloocyte membrane with the non-exchangeable phospholipid analog N-Rh-PE. Using an analogous procedure as outlined for R18, the probe can be intercalated at low temperature in the plasma membrane of cells. Warming of the labeled cells results in internalization of the probe by endocytosis, as has been shown to occur in BHK cells (19) and HepG2 cells (20). With this procedure, N-Rh-PE-labeled EVs (note that the probe is asymmetrically located, i.e. in the inner leaflet only) were isolated after in situ labeling. Subsequent incubation with unlabeled liposomes for 30 min at 37 °C, dictated by the R18 assay (Fig. 1), showed that a fusion product was obtained with a density that was less than that of the EVs per se (Fig. 3). Furthermore, measurements of rhodamine fluorescence before and after addition of Triton X-100 revealed that relative to the fluorescence measured in fractions 4–6 (Fig. 3), the fluorescence in fractions 8–10 was decreased (approximately 50%). Although no efforts were undertaken to correlate the extent of dequenching to the extent of fusion, it is apparent that a relative dequenching, in conjunction with a shift in density, is consistent with the occurrence of dilution due to merging of labeled EVs with unlabeled liposomes.

Apart from membrane mixing, fusion should also lead to the mixing of contents, bounded by the membranes of the vesicle populations involved. For membrane vesicles of biological origin, such an approach is commonly impossible. In this case, however, the exclusiveness of the system could be exploited in that Tf is the sole substrate carried by the EVs. Therefore, EVs were isolated that contained 125I-Tf. As shown in Fig. 4, when such vesicles had been incubated with liposomes for 30 min at 37 °C, a shift to lower density occurred for the Tf in the gradient. In fact, the vesicle fraction of lower density was recovered at a density identical to that observed for the N-Rh-PE-labeled fusion product (Fig. 3). This result strongly supports the view that the shift in contents localization reliably reflects a shift due to membrane mixing as a result of fusion. Furthermore, quantification of the contents mixing can be approximated by subtracting the gradient patterns (Fig. 4B). It can thus be estimated that approximately 20% of the internal contents of the endocytic vesicles is shifted to lower density due to fusion. This number corresponds very well with the level of lipid dilution, obtained in case of the R18 assay. Thus, in a parallel experiment, R18-labeled EVs were incubated with liposomes at otherwise identical conditions. After 30 min at 37 °C, the extent of dilution was determined by measuring fluorescence

abolished, showing a residual rate of 0.46%/min, i.e. less than 10% of the rate in saline buffer (cf. curve a versus curve d in Fig. 1). Neither was substantial lipid dilution observed when the experiments were carried out at 10 °C, where an initial rate was seen of 0.15%/min, i.e. less than 5% of that obtained at 37 °C (see Fig. 7). Taken together, these experiments indicate that if these conditions reflect conditions at which fluorescence development only occurs as a result of spontaneous movement of the probe, such monomeric transfer would contribute at most 5–10% to the fluorescence development obtained at fusogenic conditions.

Although the latter observations, in particular, supported the occurrence of lipid dilution as a result of fusion, rather than transfer of R18 monomers between labeled and unlabeled membranes, several additional control experiments were carried out to corroborate this notion.

EVs and liposomes can be separated on sucrose density gradients because of a difference in density. After fusion, the fusion product will differ in composition, and hence density.
state of hydration, DOPE has a propensity to form in isolation the hexagonal HII phase. This bilayer to non-bilayer transition is mediated through formation of inverted micelles. Evidently, when as opposed membranes fuse, membrane destabilization has to occur (25), and the potentially membrane-destabilizing properties of DOPE may therefore be of relevance, irrespective of the nature of the intermediate structure, which depends on the experimental conditions. Cyclosporin A and Z-FG are known to stabilize membranes by interfering with non-bilayer transitions (26). As shown in Fig. 6, both compounds strongly inhibited the fusion of EVs with DOPE/DOPC liposomes. For both compounds, the maximal efficiency of inhibition was about 70–80% (Fig. 6). Furthermore, mass action kinetic analysis of the data obtained in the presence of the peptide reveals that the fusion rate constant decreases approximately 4-fold at these conditions, with little effect on the aggregation rate constant. Hence, the peptide interferes with the fusion step per se rather than the close approach of EVs and liposomes. The specificity of the inhibitory effect is apparent by the observation that the tripeptides Z-GGG and Z-GGA were without effect on fusion (Fig. 6). Moreover, cyclosporin A did not interfere with the fusion between EVs and PS vesicles, implying that identical kinetics were obtained in the presence or absence of the drug (not shown).

Modulators of EV Fusion—To further characterize the fusogenic properties of EV with DOPE/DOPC membranes, various parameters were examined which were thought to play a role in the overall fusion event in vivo. Addition of ATP (1 mM), GTP (1 mM), and GTPγS (20 μM) did not affect the fusion reaction, implying that the fusogenic interaction between EV and liposomes does not depend on energy and/or GTPase activity. Intriguingly, addition of cytochalasin D (final concentration 0.6 mg/ml) strongly inhibited the rate of lipid dilution by approximately 90%, while the addition of AlF4 (10 mM NaF, 20 μM AlCl3) did not affect the fusion between EVs and liposomes. When increasing concentrations of Ca2+ were included in the incubation medium (up to 5 mM), no effect on both the rate or extent of fluorescence increase was seen, suggesting that the fusion event was Ca2+ independent. Finally, fusion between EV and DOPE/DOPC liposomes is strongly dependent on temperature. As shown in Fig. 7, lowering the temperature from 37 to 14°C gradually inhibited the fusion, the initial rate becoming Virtually negligible around 14°C.

Involvement of Protein(s) in EV Fusion—In contrast to pre-treatment with N-ethylmaleimide, treatment of the endocytic vesicles with trypsin strongly inhibited the fusion activity of the vesicles. Fig. 8 shows the effect of trypsinization time on the initial rate of fusion. Treatment for 30 min with the enzyme

\[^2\] M. Vidal, S. Nir, and D. Hoekstra, unpublished observations.
resulted in an inhibition of approximately 80%. This effect would support the role of (a) protein(s) in the fusion reaction. To determine whether these proteins represented a peripheral or integral protein fraction, EVs were extensively washed with 0.1 M Na₂CO₃, pH 11. This treatment has been reported before to remove peripheral proteins from membranes (27). However, the fusogenic capacity of the vesicles was not affected after such treatment, implying that the potential protein(s) involved are of an integral nature.

**DISCUSSION**

In this work we have shown that the fusion of endocytic vesicles can be monitored in a reliable and convenient manner, using an assay based on lipid mixing. In contrast to the more laborious methods used so far, and which are largely based on immunoprecipitation techniques, kinetic and quantitative data are readily obtained by the procedure described here. To validate the application of a lipid mixing assay as a measure of fusion, it is imperative to take into account that monomeric lipid transfer may occur. Therefore, a note of caution is appropriate. As shown here, a variety of control experiments can be carried out to determine the extent to and conditions at which such a non-fusion-mediated background transfer interferes with fusion-mediated lipid dilution. Furthermore, when plotting the initial rate of fluorescence increase versus acceptor vesicle concentration (Fig. 1), a background i.e. non-fusion-mediated transfer can be derived from the intercept with the y axis (0.7–0.8%/min; not shown). Particularly at a low acceptor concentration, spontaneous transfer can contribute significantly to the overall lipid dilution. Such an approach further emphasizes the need of carefully defining the experimental conditions such that the acceptor vesicle concentration is in a range where the acceptor concentration-dependent transfer, i.e. fusion, predominates, i.e. well above 10 μM. While this article was in preparation, Mullock et al. (28) used a similar approach to characterize the fusion between late endosomes and lysosomes. This enables them to demonstrate that R18-labeled dense endosomes fused with lysosomes but not with mitochondria, unlabeled dense endosomes, or light endosomes. In the present work evidence has been obtained which supports a specific role of phospholipid in the target membrane for fusion of endocytic vesicles. This lipid appears to be unsaturated PE, which, in isolation, readily adopts a nonbilayer phase. Obviously, such a membrane destabilizing property must be intimately related to merging of apposed membranes. Our data further indicate that (a) protein(s), tightly associated with the endocytic vesicle membrane, is (are) involved in the overall process. From the literature it is evident that the number of molecules that govern intracellular fusion steadily increases. However, as far as their role has been determined, many of these factors seem to be involved in targeting and docking. To distinguish between both these events and fusion per se, we have simplified the cell-free system by using liposomes as target membranes. The process that was reconstituted in our study represents in essence the fusion susceptibility of the endocytic vesicles toward the inner leaflet of the reticulocyte membrane. After their biogenesis upon internalizing the Tfiron complex, the majority of the vesicles rapidly returns to the cell surface, after expulsion of the iron. Consistent with this notion is the observation (not shown) that fusion of EVs among themselves is minimal, proceeding with an initial rate of approximately 0.5%/min. These experiments were carried out at conditions identical to those at which the fusion with phospholipid vesicles was examined. It is possible, however, that fusion of the vesicles among themselves requires an advanced level of fine regulation of recognition and docking ("priming"), provided by molecular factors (e.g. rab proteins, nucleotides, and other

**FIG. 6.** A. effect of cyclosporin A and Z-IFG on EV-liposome fusion. EVs-R18 were equilibrated in saline buffer at 37 °C. DOPC/DOPE (6:4) liposomes (200 μM) preincubated with cyclosporin A or Z-IFG (30 μM) for 5 min at 20 °C were then added. Z-GGG and Z-GGA were incubated with the liposomes in a similar manner and used as control peptides. Initial rates of fusion were calculated as described under Experimental Procedures. B. Concentration curve of fusion inhibition by Z-IFG. Fusion measurements were carried out as described in A with liposomes preincubated with increasing concentration of Z-IFG. Addition of the same amounts of ETOH did not affect initial rates of fusion. Data are presented as mean ± S.E.

**FIG. 7.** Temperature dependence of EV-liposome fusion. EVs-R18 were equilibrated in saline buffer at the indicated temperatures, and 200 μM of liposomes of DOPC/DOPE at a molar ratio of 6:4 equilibrated similarly were added. Initial rates of fusion were determined as described under Experimental Procedures.

**FIG. 8. Effect of EV trypsinization on fusion.** EVs-R18 (5 μL) were treated with trypsin (250 μg/mL) in saline buffer (100 μL) for the indicated time at 37 °C. The reaction mixture was then mixed with liposomes (DOPC/DOPE, 6:4) and fusion was monitored as described in Fig. 1. Results are mean ± S.E.
cytoplasmic components) that were not included in the incubation medium. It should also be noted that the EVs fused with liposomes, provided that an excess of target membranes was used, i.e. to supersede the targeting and docking steps.

Considering the membrane topology of the major fusion event, the susceptibility of the EVs for PE in the target membrane would be in accordance with the asymmetric localization of this phospholipid. The specificity of this requirement is further emphasized by the observation that PS vesicles represented a relatively poor target membrane for the EVs. This is remarkable since fusion of a variety of biological membranes, including sea urchin egg secretory granules (22), rabbit liver Golgi membranes (21), viruses (23), and spermatozoon (24), is usually strongly facilitated when PS is included in the target membrane. The effect of PE could be explained by the low hydration of its polar headgroup compared to the repulsive hydration layer associated with the headgroup of PC. Thus PE would provide a more hydrophobic bilayer surface, susceptible to energetically more favorable interbilayer interactions. On the other hand, not only could PE facilitate the close approach of bilayers, the lipid may also be directly involved in the merging process. In this context, PE can form the hexagonal HII phase (29), the formation of which involves the development of nonlamellar structures thought to be relevant as intermediates in membrane fusion (25). Agents such as the tripeptide Z-FGG or the immunosuppressive agent cyclosporin A have been shown to inhibit formation of the HII phase (30). In fact it has also been shown that these compounds prevent the formation of highly curved phospholipid surfaces (31), a membrane property that, in analogy to highly curved small unilamellar vesicles, acts as a strong promoter of membrane fusion. Either one or both of these properties may be relevant to the fusogenic properties of the EVs. Evidence concerning the specificity of the effectors was provided by experiments showing that nonrelated tripeptides do not affect fusion, while cyclosporin does not affect fusion with PS vesicles. Both the amino acid composition of the peptide and the carbonylenzoyl group appear to be important structural requirements for the inhibitory effects of peptides on membrane fusion (32). It has also been reported that (Z)-Gly-Phe-amide, but not (Z)-Gly-aminodecyl, inhibits the recycling of asialoglycoproteins (33). The effect was suggested to be related to the involvement of metalloprotease activity, which was inhibited by the peptide, in the fusion event of recycling vesicles with the plasma membrane. Moreover, it has been shown recently that the dipeptide (Z)-Gly-Phe-amide inhibits homotypic endosome fusion, whereas the control dipeptide (Z)-Gly-aminodecyl was without effect (34). Although the dipeptide concentration used (3 mM) was much higher than in the present work, the results of these studies are consistent with the present data and strengthen the relevance of the semi-artificial system as described here to determine molecular parameters that govern endocytic vesicle fusion. Since enzyme activity cannot play a role in our system, we therefore propose that properties related to nonbilayer transitions, as reflected by the molecular properties associated with DOPE, are the driving force for EV fusion and that agents that interfere with these bilayer perturbing properties, such as distinct di- and tripeptides, will inhibit the fusion event. In this context, it is finally relevant to note that exocytosis in cultured cell lines as well as the exocrine pancreas from rat, can be catalyzed by cyclosporin A, presumably at the level of the fusion event (35, 36).

At present the nature of the DOPE structure that promotes the EV fusion event is unclear. Formation of the HII phase represents a stable molecular structure reached at equilibrium. This structure is not likely formed in mixed bilayer systems and is, conceptually, difficult to reconcile with such a dynamic, transient event as membrane fusion. A local recruitment, at sites of interbilayer contact, of lipid molecules susceptible to bilayer departure seems possible, and protein-triggered changes in polymorphic properties of PE species have been reported (see e.g. Ref. 37). The dynamics of such properties may result in lipid scrambling (38) and leakage of vesicles contents, as shown for virus-liposome fusion (39, 40). A protein triggered change in PE polymorphic properties may represent an important aspect of the trypsin-sensitive, but N-ethylmaleimide-insensitive, protein (fraction) that we identified, and which appears to be tightly (transmembrane?) associated with the EV membrane. For endosome-endosome fusion, involvement of a similar N-ethylmaleimide-resistant, trypsin-sensitive protein has been reported (41). However, this protein was peripherally associated with the endosomal membrane, since alkaline treatment effectively abolished endosome fusion. The protein involved is not AP-2, the clathrin assembly protein, which can induce aggregation of stripped coated vesicles (42) and, in purified form, the aggregation of liposomes. We observed, however, that, although AP-2 is present on the EV surface, addition of anti-AP-2 antibodies did not inhibit EV-liposome fusion. Furthermore, various rab proteins are associated with EVs (14). Although not defined yet, a direct partitioning in the environment of such proteins seems unlikely, their role being primarily restricted to events ("priming") that precede the fusion step (9, 10, 43). Concerning the functional role of the protein(s) identified in the present study, it will be of interest to determine whether this role is exclusively limited to fusion or that this protein (or perhaps additional ones) is involved in some aggregation activity as well. Since fusion is completely inhibited when the incubation is carried out in an isosmotic sucrose-containing medium, electrostatic interactions seem relevant, suggesting a rather nonspecific mode of interaction. Concerning the characteristics of the potential fusion protein, it is at present interesting to note that this protein apparently acts without the need of GTP or its hydrolysis. Based on fusogenic work involving biological membranes such as viruses or spermatozoa, it is then pertinent to point out that also in those cases, fusion proceeds in an energy-independent manner. In fact, existing evidence supports the notion that GTP hydrolysis is not required for endosome fusion. The characteristics of the fusion protein associated with EVs are currently being examined.

REFERENCES
