J. Membrane Biol. 142, 35–42 (1994)

The Journal of
Membrane
Biology

© Springer-Verlag New York Inc. 1994

A Fusogenic Protein from Rat Brain Microsomal Membranes: Partial Purification and Reconstitution into Liposomes

M. Rakowska[1,2], **J. Zborowski**[2], **L. Corazzi**[1]

[1]Institute of Biochemistry and Medical Chemistry, The University of Perugia, Via del Giochetto, 06100 Perugia, Italy
[2]Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, Poland

Received: 7 January 1994/Revised: 26 May 1994

Abstract. The procedures for purification and reconstitution of rat brain microsomal membrane protein that causes fusion of liposomes at acidic pH are described. A 1,860-fold purification was achieved, starting from the detergent-solubilized microsomal membranes. The fusion process was assayed spectrofluorimetrically by monitoring the formation of terbium-dipicolinic acid complex (Wilschut, J. et al. 1980. *Biochemistry* **19:**6011–6021) evoked by the protein after mixing of two populations of liposomes. The fusogenic activity of the protein inserted into the membrane of Tb^{3+}-containing vesicles was found to be strongly dependent on phospholipid composition and was higher in vesicles enriched with exogenous phosphatidylserine, phosphatidylglycerol and phosphatidylethanolamine than in those prepared with an excess of phosphatidylcholine. The vesicles enriched in negatively charged phospholipids were bound to Concanavalin A coupled to Sepharose-4B and could be released from this column only in the presence of a high concentration of α-methylmannopyranoside and detergent, indicating a glycoprotein nature of the fusogenic protein. Furthermore, these data show that protein inserted into membrane has its oligosaccharide chains exposed to the environment.

Key words: Fusogenic protein — Glycoprotein — Brain — Microsomes — Liposomes — Membrane reconstitution

Introduction

Intracellular membrane fusion is a crucial step in many cellular activities such as endocytosis and transfer of components among cellular organelles. A number of studies on artificial phospholipid membrane systems have been made to gain insight into the mechanism and role of biological membrane fusion (Düzgünes et al., 1987). Several factors can induce fusion. Divalent cations (Ohki, 1982; Bental et al., 1987; Leckband, Helm & Israelachvili, 1993), as well as a variety of peptides and proteins (Surewicz et al., 1986; Glushakova et al., 1992), have been shown to participate in a fusion process. The fusogenic activity of viral proteins has been thought to depend on their acidic amino acid moiety, protonated following acidification of the medium (Murata et al., 1992). The role of H^+ has been investigated by several reports. Protons can also promote the fusion between liposomes and viral structures (Wunderli-Allenspach & Ott, 1990). Liposomes are induced to fuse upon acidification in systems in which lectin mediates the contact between vesicles (Kim & Kim, 1986). The endoplasmic reticulum is also involved in several events requiring membrane fusion. We reported that microsomal protein mediates a pH-dependent fusion of liposomes to rat brain microsomes (Pistolesi, Corazzi & Arienti, 1992). This fusion is trigged at acidic pH and is dependent on the nature of liposomes. Moreover, the pretreatment of microsomes with pronase or with a reagent for carboxylic groups inhibits fusogenic activity.

This paper describes a procedure for the partial purification and reconstitution of the pH-dependent fusogenic protein from rat brain microsomal membranes into liposomes. The dependence of fusogenic activity on the composition of lipid mixtures used for protein reconstitution is also reported.

Correspondence to: L. Corazzi

Materials and Methods

Materials

HEPES, Thesit, *n*-octylglucoside and MES were produced by Boehringer-Biochemie (Mannheim, Germany). Sephadex G-50, Con

A-Sepharose and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). DEAE-Biogel A, Bio-Beads SM2 and SDS-polyacrylamide gel electrophoresis reagents were from Bio-Rad (Richmond, CA). Pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) and $TbCl_3 \cdot 6\ H_2O$ and α-methylmannopyranoside were purchased from Aldrich Chemie (Steinheim, FRG). Octadecyl rhodamine B chloride (R_{18}) was from Molecular Probes (Eugene, OR). L-α-phosphatidylserine and other lipids were obtained from Sigma Chemical (St Louis, MO). Lipids from rat liver were extracted as described by Folch, Lees and Sloane-Stanley (1957).

Preparation of Microsomes

Charles River male rats (weighing 150–200 g) were used to prepare brain microsomes, following the routine procedure (Corazzi et al., 1986). Membranes were resuspended in cold 0.32 M sucrose, 2 mM HEPES, pH 7.4 (S/H buffer) and used for the solubilization with Thesit.

Preparation of DPA- and Tb^{3+}-loaded Liposomes

Suitable amounts of total liver lipids were dissolved in chloroform/methanol (2:1, by vol) and the solvent was removed with a nitrogen stream. DPA-loaded liposomes were prepared by suspending the lipids (about 1.5 μmol/ml) in a buffer containing 2 mM L-histidine, 2 mM HEPES and 150 mM dipicolinic acid, pH 7.4. Suspensions were sonicated to clearness in a MSE sonicating apparatus. Tb^{3+}-loaded liposomes were prepared by the same procedure, with the exception that lipids were suspended in a buffer containing 2 mM L-histidine, 2 mM HEPES, 15 mM $TbCl_3$ and 150 mM sodium citrate, pH 7.4. Nonencapsulated DPA and Tb^{3+} were removed by filtering the vesicles through a Sephadex G-50 column (Corazzi et al., 1989). Leakage of the probes from the vesicles was negligible at least for 2 hr, as determined by adding free Tb^{3+} (0.75 μM) to DPA-loaded liposomes or free DPA (5 μM) to Tb^{3+}-loaded liposomes (Rosenberg, Düzgünes & Kayalar, 1983).

Assay of Fusion

After each purification step the protein's ability to promote liposome fusion was tested. The extent of fusion was measured by the Tb/DPA method (Wilschut et al., 1980). The fusion assay was performed in a cuvette containing 2 ml of 0.32 M sucrose, 20 mM MES, pH 5.0, Tb^{3+}-liposomes (50 nmol lipid) and DPA-liposomes (50 nmol lipid). To start fusion, we added an aliquot of the protein. Mixing of the aqueous content of vesicles following fusion resulted in the formation of Tb/DPA fluorescent complex (Wilschut et al., 1980). This was monitored in a MPF-3 Perkin Elmer apparatus. Excitation and emission wavelengths were set at 276 and 545 nm, respectively. A cut-off filter (UV 31) was inserted to eliminate the contribution of light scattering. Slits were 10 nm for both excitation and emission. The value of maximal fluorescence with each Tb^{3+}- and DPA-liposome preparations was determined in the presence of 0.03% Thesit. The obtained values allowed comparison of different liposomal preparations used in the experiments. The fusogenic activity is expressed in arbitrary units of elicited fluorescence. In the case of reconstitution experiments, the fusogenic protein was inserted into the membrane of Tb^{3+}-loaded vesicles. Fusion activity was monitored after mixing the vesicles with DPA-liposomes. In some experiments, fusogenic activity was measured using the Octadecyl Rhodamine (R_{18}) method which has been largely used to study the intermixing of membrane components (Hoekstra et al., 1984). Experiments carried out using R_{18} as fluorescent probe were compared to that performed with the Tb/DPA method. R_{18} was inserted into liposomal preparations as described in detail in our previous papers (Corazzi et al., 1989; Pistolesi et al., 1992). The fusion process was then monitored measuring the relief of self-quenching of the fluorescent probe at 580 nm (excitation 560 nm), which followed the mixing of R_{18}-loaded and unloaded liposomes in the presence of fusogenic protein.

Purification Procedure

Solubilization of Microsomes

Microsomes suspended in S/H buffer (120 mg protein, 10 ml) were added to 10 ml of a 2% Thesit solution. After incubation for 10 min at 5°C, the nonsolubilized residue was removed by centrifugation (105,000 × *g*, 1 hr).

Ammonium Sulfate Treatment

Ammonium sulfate was gradually added to supernatant to 30% saturation. The solution was stirred for 10 min and then centrifuged (105,000 × *g*, 1 hr). After centrifugation, two phases were obtained: the upper phase was a thin film containing Thesit, lipids and a portion of hydrophobic proteins, whereas the lower phase contained protein (about 20% of the total) in the ammonium sulfate solution. The upper phase was discarded and the lower phase was dialyzed extensively *vs.* S/H buffer and concentrated utilizing collodion-bags and a Sartorius apparatus.

DEAE-Biogel A Column Chromatography

The dialyzed solution after ammonium sulfate treatment (20 mg protein, 1 ml) was applied to a DEAE-Biogel A column (1 × 10 cm) pre-equilibrated in S/H buffer, pH 7.4. Elution was performed with three discontinuous steps of S/H buffer containing 0.1, 0.2 and 1 M NaCl. The active protein, eluted at 0.2 M NaCl, was dialyzed *vs.* a solution containing 10% glycerol, 2 mM HEPES, pH 7.4 and concentrated as above.

Affinity Chromatography

Concanavalin A coupled to Sepharose 4B pre-equilibrated in 0.1 M acetate buffer (pH 6.0) containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ was used to pack a small column (1 × 6 cm). The column was washed once with three-column volumes of a solution containing 20 mM HEPES pH 7.4. An aliquot (1 ml) of the active peak eluted from DEAE-Biogel A (4 mg protein) was layered on top and washed with the following buffers: (a) 20 mM HEPES pH 7.4; (b) 20 mM HEPES, 1 M NaCl, pH 7.4. The bound fraction containing the protein with fusogenic activity was desorbed by eluting with 2 mM HEPES, 1 M NaCl, 1 M α-methylmannopyranoside in 10% glycerol, pH 7.4.

Sephacryl S-200 Column

Solution from the last step, dialyzed *vs.* S/H buffer and concentrated as described, was applied to a Sephacryl S-200 column (1 × 70 cm) previously calibrated with standard proteins. The column was pre-equilibrated with S/H buffer containing 0.2 M NaCl (pH 7.4). The same buffer was used as eluent. Three peaks were obtained. The first corresponded to the void volume of the column. The bulk of fusogenic

Table 1. Purification of a fusogenic protein from rat brain endoplasmic reticulum

	Protein (mg)	Activity (fluorescence units)	Recovery (%)	Specific activity (fluorescence units/ mg protein)	Purification factor
Microsomes	120	180	100	1.5	1
$(NH_4)_2SO_4$	19.5	163	91	8.3	5.5
DEAE Biogel A	4.3	95	53	22	15
Con A	0.16	67	37	419	279
Sephacryl S-200	0.01	28	16	2,800	1,860

The fusogenic activity is expressed in arbitrary fluorescence units measured 10 min after mixing of Tb^{3+}- and DPA-loaded liposomes. For each purification step, the estimated fluorescence intensity was in the sphere of linearity *vs.* fusogenic protein concentration. In some instances the fusion activity was assayed with the R_{18} method. A close correspondence between the results obtained by both assays was always found.

activity appeared with the third peak corresponding to a M_r of 45,000–70,000.

Preparation of Liposomes with Reconstituted Fusogenic Protein

Reconstitution of the fusogenic protein into lipid bilayer was performed by co-solubilization of lipids and proteins with octylglucoside followed by dialysis of the solubilized material *vs.* S/H buffer in the presence of Bio-Beads SM-2. More in detail, 2 mg of lipids in chloroform were dried under a nitrogen stream and treated with 0.5 ml of S/H buffer containing 80 mM octylglucoside. After lipid solubilization, the fusogenic protein (0.5 ml, 125 or 50 μg) was added and the suspension was kept on ice for 10 min. The mixture was then poured into a dialysis bag and dialyzed at 4°C against 100 ml of S/H buffer. The velocity of the dialytic step was increased adding about 0.5 g of Bio-Beads SM-2 to the external medium. The medium and the Bio-Beads were replaced after 2 hr and dialysis was continued up to 16 hr. To estimate the formation of the vesicles and their fusogenic activity, many experiments were performed preparing Tb^{3+}-loaded reconstituted vesicles. In those cases lipid solubilization was performed by adding 0.5 ml of S/H buffer (pH 7.4) containing 80 mM octylglucoside, 1 mM histidine, 150 mM sodium citrate and 30 mM $TbCl_3$. The fusogenic protein was added thereafter and the detergent removed by dialysis. To remove nonencapsulated Tb^{3+}, the vesicles were filtered through a Sephadex G-50 column as described above.

Density Gradient Centrifugation of Reconstituted Liposomes

A sample (1 ml) of reconstituted liposomes was layered on top of a continuous gradient of 0.4–1.2 M sucrose (4 ml). The sample was centrifuged at 242,000 × *g* in a Beckman VT165 vertical rotor for 4 hr at 4°C. After centrifugation, 0.5 ml fractions were collected by piercing the bottom of the tube.

Affinity Chromatography of Reconstituted Liposomes

Reconstituted liposomes (1 ml) were loaded on a concanavalin A column equilibrated with 20 mM HEPES, 0.2 M NaCl (pH 7.4). After several washings, the bound liposomes were eluted with the same buffer containing 0.5 M α-methylmannopyranoside and 4 mg/ml of octylglucoside. The material eluted from the column was dialyzed against S/H buffer and analyzed for the content of lipid and protein.

Analytical Procedures

SDS-polyacrylamide slab gel electrophoresis was performed according to Laemmli (1970) with the modification that the separation gel contained 11% acrylamide. The gel was run in the Bio-Rad Mini-protean apparatus. Staining was performed using the silver nitrate method (Giulian, Moss & Greaser, 1983). Protein was determined as described by Bradford (1976). Phospholipid phosphorus was assayed after digestion with perchloric acid (Bartlett, 1959).

Abbreviations

HEPES: 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; MES: 2-(*N*-morpholino)-ethanesulfonic acid; S/H buffer: sucrose-HEPES buffer (320 mM sucrose + 2 mM HEPES, pH 7.4); Thesit:dodecyl-poly(ethylenglycolether)$_9$; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PS: phosphatidylserine; CL: cardiolipin; Chol.: cholesterol.

Results

Partial Purification of the Protein Capable of Inducing the Fusion of Liposomes at Acidic pH

Table 1 summarizes a typical purification procedure of microsomal rat brain protein capable of inducing liposome fusion when the pH is brought below 7.0. Treating membranes with 1% Thesit solubilized about 80% of total membrane components. Nonsolubilized material, removed by centrifugation, did now show fusogenic activity. Addition of $(NH_4)_2SO_4$ up to 30% saturation to the solubilized material produced, after centrifugation, a detergent/lipid/protein gelatinous upper phase and a lower phase containing the fusogenic protein in ammonium sulfate solution. It is worth noting that ammonium sulfate can induce phase separation of the detergent solution into detergent-rich and detergent-depleted phases. Analysis of the lipid content of the detergent-rich and detergent-depleted phases showed that lipids were completely removed from the detergent-depleted

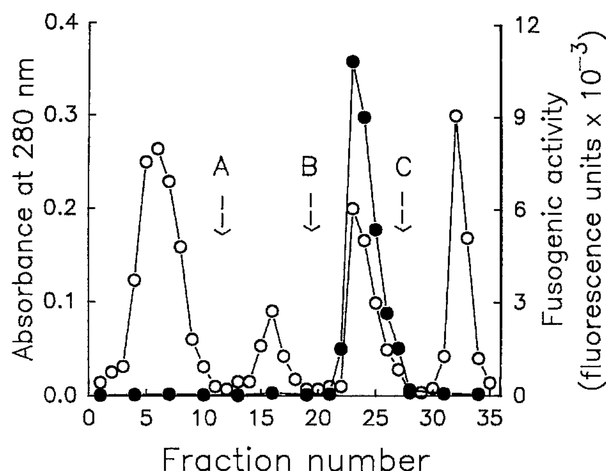

Fig. 1. DEAE-Biogel A chromatography of a fusogenic protein from rat brain microsomal membranes. Dialyzed and concentrated solution after ammonium sulfate treatment (20 mg protein, 1 ml) was applied to DEAE Biogel A column (1 × 10 cm) equilibrated with S/H buffer, pH 7.4. Protein was eluted with the same buffer containing increasing concentrations of sodium chloride: buffer A, 0.1 M; buffer B, 0.2 M; buffer C, 1 M. Fusogenic activity was measured with proper aliquots of each eluted fraction. (○—○), protein content; (●—●), fusogenic activity.

phase, which in turn contained about 20% protein and 91% fusogenic activity.

Figure 1 shows the elution profile of the DEAE Biogel A column. Fusogenic activity was eluted as a single peak with 200 mM NaCl in S/H buffer, pH 7.4. The fusogenic peak contained 22% of applied proteins and about 58% of the loaded activity. The specific activity was raised about three times compared to that recovered from the previous step.

The fusogenic protein was retained by the concanavalin A column, suggesting that it is a glycoprotein. In fact, a peak containing 70% of activity was released from the column after passage of α-methyl-mannopyranoside, representing about 4% of loaded proteins. The specific activity of the fusogenic protein increased about 19 times compared to the previous step (Table 1). The intensity of fluorescence, elicited upon addition of the fusogenic protein to a mixture of Tb^{3+}- and DPA-loaded liposomes, was linear up to 0.1 μg protein. In a range of protein concentration linearity a plateau of fluorescence was reached after 10 min.

The purification factor increased noticeably (Table 1) following passage through the Sephacryl S-200 column, which shifted in three peaks the material eluted from the Con A column. The bulk of activity was found in the third peak, although a part of it was also found in the first peak corresponding to the void volume of the column. The tendency to aggregate in solution can explain the nonlinearity of the fusogenic activity *vs.* protein concentration at high amounts of protein and also the presence of a part of fusogenic material in the void volume of the Sephacryl S-200 column. In fact, the chromatographic step performed with the Sephacryl S-200 column gave a low recovery of the total fusogenic activity, although it gave a protein of a very high specific activity. The material eluted in this step was difficult to handle, because of the low protein content which made difficult its accurate determination. Therefore, in all reconstitution experiments a fusogenic protein eluted from Con A column was used.

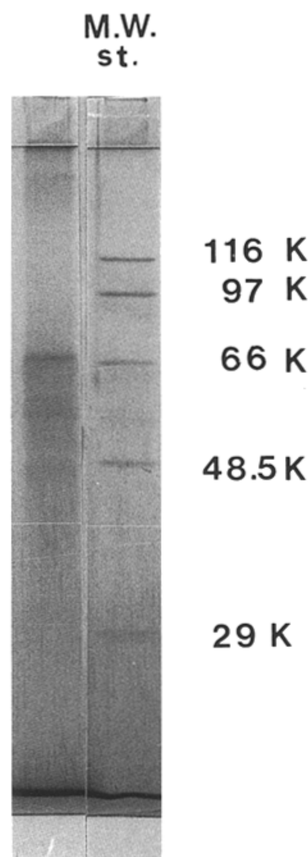

Fig. 2. SDS-polyacrylammide gel electrophoresis of a purified fusogenic protein preparation. The active fractions of the third peak of a Sephacryl S-200 column were pooled, and an aliquot (1 μg protein) was submitted to slab gel electrophoresis as described in Materials and Methods. β-Galactosidase (116,000), phosphorylase b (97,000), serum albumin (66,000), fumarase (48,500) and carbonic anhydrase (29,000) were used as standards for molecular weight calibration.

Figure 2 shows the electrophoretic analysis of the active peak eluted from Sephacryl S-200. Four to five bands, corresponding to approximate molecular weights of 40,000–70,000 D were seen after silver staining.

Stability of the Solubilized Protein

The protein is stable in S/H solution. Dialytic removal of sucrose resulted in a complete loss of activity. When necessary, sucrose was replaced by 10% (w/v) of glyc-

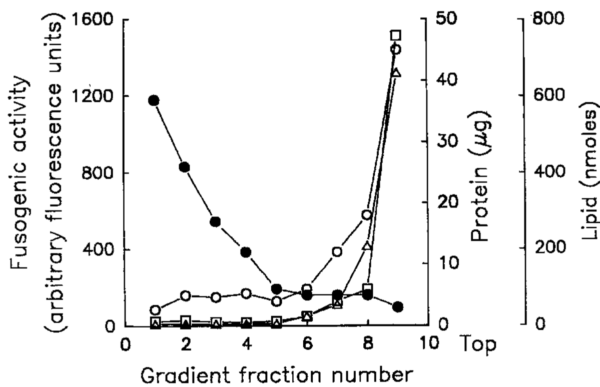

Fig. 3. Centrifugation of the reconstituted vesicles on sucrose density gradient. A sample (1 ml) of reconstituted vesicles was layered on top of a discontinuous gradient of sucrose and centrifuged as described in Materials and Methods. Fractions collected after centrifugation were tested for fusogenic activity (△—△), protein (○—○) and lipid (□—□) contents. In control experiment, the fusogenic protein not inserted into liposomes was centrifuged in the same experimental conditions (●—●).

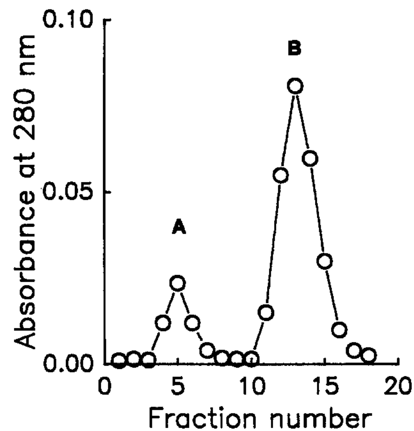

Fig. 4. Elution profile of the reconstituted vesicles with inserted fusogenic protein from Con A-Sepharose column. Fusogenic protein was reconstituted into liposomes made of PC:PE:PS:CL, (60:25:10:5, weight ratio) with a lipid protein weight ratio of 16:1. Peak *A* (unbound material) was eluted with 20 mM HEPES and 0.2 M NaCl (pH 7.4). Peak *B* (bound material) was eluted with the same buffer containing 0.5 M α-methylmannopyranoside and 4 mg/ml of octylglucoside.

erol. No appreciable loss of activity was found after 1 month of storage at −18°C.

Reconstitution of Fusogenic Protein into Liposomes

The glycoprotein fraction eluted from the concanavalin A column was reconstituted into liposomes. The protein fraction was mixed with lipids (protein/lipid weight ratios of 1:16 or 1:50). Octylglucoside was used to dissolve both the proteins and the lipids, and reconstitution was accomplished by dialytic removal of detergent in the presence of polystyrene beads as described in Materials and Methods. Pure phospholipids or different mixtures were used throughout this work. The removal of detergent from the detergent/lipid/protein-mixed micelles resulted in the formation of lipid vesicles with reconstituted protein. This was proved by the centrifugation of reconstituted vesicles on sucrose density gradient. Figure 3 shows that all fusogenic protein was found together with the lipid in the upper part of the gradient. In a parallel experiment using unreconstituted material, the protein was recovered in the lower part of the gradient. Since no protein was detected at this position using reconstituted protein, we can conclude that all fusogenic protein is lipid associated in liposome preparations. That vesicles have been formed was further demonstrated by the presence of trapped Tb^{3+}, previously added to the mixture of detergent, protein and lipid, as described in Materials and Methods. The amount of Tb^{3+} trapped into these liposomes could be observed thereafter in the assay with DPA-containing liposomes and proper concentration of disrupting detergent. The reconstituted vesicles with inserted fusogenic protein and containing Tb^{3+} were utilized in the subsequent experiments to measure the fusogenic activity of the protein.

The fusogenic activity of reconstituted vesicles was tested with the Tb/DPA method, although we checked also the intermixing of bilayers with the R_{18} assay. Results obtained with both methods were comparable. Therefore, as shown by the two independent assays, the fusogenic protein involves intravesicular reaction of Tb^{3+} and DPA and intermixing of membrane components.

The fusion process trigged by the fusogenic protein was not leaky. This was monitored by observing that fluoresence was not elicited when Tb^{3+}-loaded liposomes were induced to fuse in the presence of free DPA.

Binding of Reconstituted Vesicles to Concanavalin A

Evidence that fusogenic protein is a glycoprotein was deduced by the high capability of the protein to bind to a Con A-Sepharose column. It was therefore expected that reconstituted vesicles should be held by concanavalin A if the proteins were inserted into the lipid bilayer with the sugar moiety exposed on the outer surface of liposomes. Our results indicate that the binding of reconstituted vesicles to concanavalin A was dependent on the nature of the lipids used for reconstitution. The lipid composition of the liposomes was varied by changing the initial composition of the lipid/protein/detergent mixtures. The initial composition of lipids and proteins in detergent mixture was identical to the final composition in liposomes as checked by TLC for lipids and by gel electrophoresis for proteins. Figure 4 shows the elution

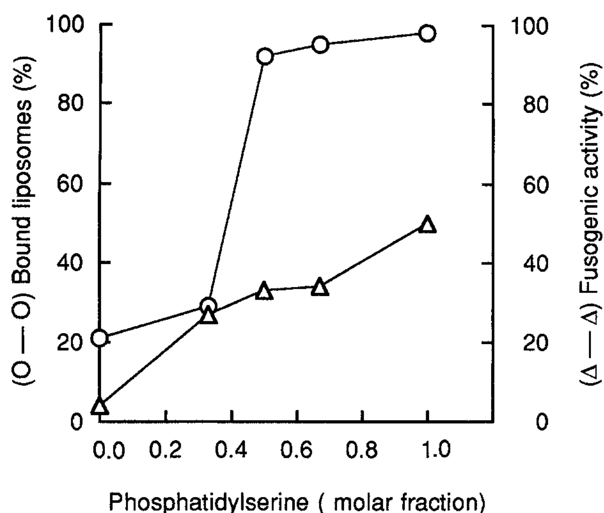

Fig. 5. Extent of binding to concanavalin A and fusogenic activity of reconstituted vesicles made of PC and PS. Fusogenic protein was reconstituted into vesicles made of PC with increasing amounts of PS. The data are expressed as a percentage of bound liposomes (determined from lipid phosphorus content) and as a percentage of maximal fluorescence (obtained in the presence of 0.03% Thesit).

Table 2. Binding of reconstituted liposomes to concanavalin A Sepharose-4B column and fusogenic activity of the vesicles with different lipid compositions

Lipid (weight ratio)	Protein/lipid (weight ratio)	Bound liposomes[a] (%)	Fusogenic activity[b]
PC	1:16	21	4.0
PC/PE (1:1)	1:16	55	15.0
PC/PE/PS (3:2:1)	1:16	65	30.0
PC/PE/PS/Chol (3:2:1:1.5)	1:16	73	19.0
PG	1:16	99	82.5
PS	1:16	98	50.0
PC	1:50	5	2.1
PC/PE (2.4:1)	1:50	19	3.2
PC/PE/PS (3:2:1)	1:50	13	7.0
PC/PE/PS/Chol (3:2:1:1.5)	1:50	27	3.6

[a] Data are expressed as a percentage of the total lipid phosphorus retained by Con A column.
[b] The fusogenic activity is expressed as a percentage of maximal fluorescence (obtained in the presence of 0.03% Thesit) and refers to plateau fluorescence values reached 10 min after the mixing of Tb^{3+}-reconstituted liposomes and DPA-loaded liposomes. DPA-loaded liposomes were prepared from total rat liver lipids.

profile of reconstituted liposomes made of PC:PE:PS:CL (60:25:10:5, weight ratio) and the fusogenic protein (lipid/protein weight ratio, 16:1) passed through a Con A-Sepharose column. Peak *A* represents the fraction of reconstituted vesicles that were not bound. Peak *B* is the fraction which was bound and eluted with 20 mM HEPES, 0.2 M NaCl (pH 7.4) containing 0.5 M α-methylmannopyranoside and 4 mg/ml of octyglucoside. The inclusion of detergent was necessary to obtain a quantitative desorption of the bound protein. Fifteen percent of both added protein and lipid was found in peak *A* whereas about 85% was seen in peak *B*.

After dialytic removal of detergent from peak *B*, both peaks were tested for their fusogenic activity. The activity was found in peak *B* but not in peak *A*. However, when liposomes from peak *A* were solubilized with Thesit and protein was recovered by ammonium sulfate treatment and centrifugation, the fusogenic activity reappeared. It may be worth noting that these results were confirmed with all kinds of reconstituted liposomes. When reconstituted liposomes were formed from pure PC (protein/lipid weight ratio, 1:16), only about 20% of lipid and protein was found to be bound to concanavalin A. On the other hand, vesicles made from pure PS were completely retained by the column. Figure 5 shows the extent of binding of reconstituted vesicles made of PC and PS. Vesicle binding increased to 95% when the molar fraction of PS increased from 0.33 to 0.5, suggesting that the sugar moiety of glycoprotein is exposed in a way that permits its interaction with concanavalin A. It is interesting to observe, however, that fusogenic activity of the reconstituted protein was almost absent in PC vesicles and increased noticeably when PC was gradually replaced by PS.

Table 2 shows the effect of lipid composition at various protein/lipid ratios on the fusogenic activity and the interaction of reconstituted glycoprotein with the Con A column. As shown in this table, the extent of fusogenic activity and the binding of reconstituted vesicles to concanavalin A is increased at a lower lipid/protein ratio in all cases.

The nature of lipids also influenced both fusogenic activity and binding. Indeed, substitution of PC in reconstituted vesicles with PE, or even better with PS, increased fusogenic activity and binding. The binding behavior of PG vesicles was similar to PS ones, but fusogenic activity of the protein was higher than that of PS vesicles. The addition of cholesterol to PC/PE/PS vesicles resulted in increased binding and decreased fusogenic activity.

Discussion

In a previous paper (Pistolesi et al., 1992) we presented evidence that rat brain endoplasmic reticulum protein can promote fusion of vesicles at acidic pH. Treatment with ammonium sulfate of the Thesit-solubilized material produced phase separation into detergent-rich and detergent-depleted phases. Such behavior already has been described for Triton X-100 and other detergents (Parish et al., 1986). In our conditions, the fusogenic protein was recovered in the detergent-depleted phase, indicating its marked hydrophilic feature. It has been

suggested that, due to their detergent-binding properties, integral membrane proteins partition into the detergent-rich phase (Bordier, 1981); however, some exceptions have been observed, and this should be the case of certain glycoproteins (Burgisser & Matthieu, 1989). The partitioning behavior of a protein molecule depends upon a number of factors, such as its ability to intercalate into detergent micelles and the nature and size of the hydrophilic portion of the molecule. Therefore, the inability of fusogenic protein to partition into the detergent-rich phase does not exclude the possibility that it is an integral membrane glycoprotein. The fusogenic activity was eluted from a DEAE-Biogel A column as a single peak at medium ionic strength (Fig. 1), and this should correspond to a protein with a certain number of residual carboxylic groups. Indeed, we found that carboxylic groups in a protonated form are involved in the mechanism promoting fusogenic trigging (Pistolesi et al., 1992). It is therefore suggested that the fusogenic protein becomes more hydrophobic by neutralization due to protonation of the carboxylic groups at acidic pH. Consequently, the protein aggregates the vesicles and interacts strongly with lipid bilayers to cause fusion. This mechanism has been proposed for the fusogenic activity of some viral glycoproteins (Murata et al., 1992).

Passage through a Con A column is a crucial step in the purification process, as it was visualized by a high purification factor. At the same time, the elution of the fusogenic protein with a very high concentration of α-methylmannopyranoside from this column supported the previous view of its glycoprotein nature. Furthermore, we recently observed that microsomal membranes treated with *N*-Glycosidase F recombinant lost their fusogenic properties (*unpublished results*).

The fusogenic protein eluted from the Con A column formed rosette-like structures in solution, as seen by negative-staining electron microscopy (*not shown*). The formation of rosette-like structures is a further demonstration of the amphipathic nature of the fusogenic protein. Similar micellar structures have been observed for the isolate envelope glycoprotein of viruses (Petri & Wagner, 1979).

When the fusogenic protein was reconstituted into phospholipid vesicles, remarkable effects were observed, depending on the nature of lipids and on the protein/lipid ratio (Table 2). Seddon (1990) suggested that the mechanism of fusion might involve a phase change in the lipid bilayer and its destabilization with the formation of inverted micelles. This event involves that repulsive hydration forces must be overcome. Consequently, the strong hydrophilic nature of the PC headgroup means that this class of phospholipids does not in general form any nonlamellar phase. However, factors promoting fusion of PC vesicles have been described (Turnois et al., 1990). In our hands, at acidic pH, PC vesicles fuse to rat brain microsomal membranes (Pistolesi et al., 1992), or to liposomes in the presence of fusogenic protein solubilized from microsomes (*not shown*). On the contrary, when the fusogenic protein is reconstituted in PC vesicles, fusogenic activity is negligible. We observed that the sugar portion of the fusogenic glycoprotein is essential for its activity. Moreover, it is known that the conformational order of a protein into the lipid bilayer is dependent on the type of phospholipids (Keller, Killian & de Kruijff, 1992). Therefore, we can speculate that in PC vesicles fusogenic protein is inserted in such a way that it hides the sugar moiety. Further support to this hypothesis comes from the limited binding of reconstituted PC vesicles to concanavalin A (Table 2).

The vesicles made with PE can fuse easily because of the spontaneous tendency to adopt hexagonal phase (Cullis et al., 1985). Also, PS-enriched vesicles exhibit hexagonal phase transition in the pH range around its pKa value (de Kroon et al., 1990). Interestingly, in the reconstituted vesicles containing PE, PS or PG, both the binding to concanavalin A and the fusogenic activity are increased. A correlation between the extent of binding and fusogenic activity can be made also when PS gradually replaces PC in PC-reconstituted vesicles (Fig. 5). These results indicate that PE or acidic phospholipids determine an orientation of the sugar moiety of the glycoprotein on the external side of the vesicles. A recent paper (Terzaghi, Tettamanti & Masserini, 1993) demonstrated that the interaction of human glycophorin with glycolipids could modify the exposure of carbohydrate moieties of the protein as a possible mechanism to regulate the receptor activity of glycoprotein. In general, the oligosaccharide chains of membrane glycoproteins have been found to be almost exclusively exposed to the extracellular environment. Recently, however, an intrinsic membrane glycoprotein with cytosolically oriented N-linked sugars has been demonstrated (Pedemonte, Sachs & Kaplan, 1990). In model liposomal membranes, orientation of the N-terminal hydrophobic sequence of the viral envelope glycoprotein with respect to the lipid acyl chains depends on the presence of PE in the structure (Martin et al., 1993). Our results demonstrate that various lipids may affect not only insertion but also orientation of the fusogenic protein in the lipid bilayer. This mechanism could also be responsible for the regulation of fusogenic activity of the protein.

The results reported in this paper refer to the overall fusion process, whose rate combines both the aggregation and fusion rates. Fusion has been studied as a mass action kinetic model, which describes the overall fusion reaction as a two-step sequence consisting of a second-order process of liposome aggregation followed by a first-order fusion reaction (Meers et al., 1992). By using different lipid compositions, it is possible to select the rate limiting step of the overall fusion process. More work will be necessary to address the role of the fusogenic protein in these two steps of membrane fusion.

However, we can speculate that in our experimental conditions, the kinetics of fusion should be controlled by the fusion rate constant, at least for PC-containing liposomes (Meers et al., 1988). Therefore, any effect of the protein in increasing fusion activity could be due to an enhanced membrane fusion rate.

Mr. Carlo Ricci is thanked for his skillful technical assistance. This work was supported by a grant from the Ministry of Education, Rome, Italy.

References

Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466–468

Bental, M., Wilschut, J., Scholma, J., Nir, S. 1987. Ca^{2+}-induced fusion of large unilamellar phosphatidylserine/cholesterol vesicles. *Biochim. Biophys. Acta* **898**:239–247

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**:1604–1607

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254

Bürgisser, P., Matthieu, J.M. 1989. Phase separation of myelin proteins in Triton X-114: differential behavior of myelin basic protein in purified myelin and in cultured oligodendrocytes. *Dev. Neurosci.* **11**:179–187

Corazzi, L., Fratto, G., Pistolesi, R., Arienti, G. 1989. Fusion of liposomes and rat brain microsomes examined by two assays. *J. Membrane Biol.* **112**:123–129

Corazzi, L., Porcellati, G., Freysz, L., Roberti, R., Arienti, G. 1986. Ethanolamine base-exchange reaction in rat brain microsomal subfractions. *J. Neurochem.* **46**:202–207

Cullis, P.R., Hope, M.J., de Kruijff, B., Verkeleij, A.J., Tilcock, C.P.S. 1985. Structural properties and functional roles of phospholipids in biological membranes. *In:* Phospholipid and Cellular Regulation, Vol. 1. J.K. Kuo, editor. pp. 1–59. CRC, Boca Raton, FL

Düzgünes, N., Hong, K., Baldwin, P.A., Bentz, J., Nir, S., Papahadjopoulos, D. 1987. Fusion of phospholipid vesicles induced by divalent cations and protons: modulation by phase transition, free fatty acids, monovalent cations, and polyamines. *In:* Cell Fusion. A.E. Sowers, editor. pp. 241–267. Plenum, New York and London

Folch, J., Lees, M., Sloane-Stanley, G.H. 1957. A simplified method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:497–509

Giulian, G.G., Moss, R.L., Greaser, M. 1983. Improved methodology for analysis and quantitation of proteins in one-dimensional silver-stained slab gels. *Anal. Biochem.* **129**:277–287

Glushakova, S.E., Omelyanenko, V.G., Lukashevitch, I.S., Bogdanov, A.A., Jr., Moshnikova, A.B., Kozytch, A.T., Torchilin, V.P. 1992. The fusion of artificial lipid membranes induced by the synthetic arenavirus "fusion peptide." *Biochim. Biophys. Acta* **1110**:202–208

Hoekstra, D., de Boer, T., Klappe, K., Wilschut, J. 1984. Fluorescence method for measuring the kinetics of fusion between biological membranes. *Biochemistry* **23**:5675–5681

Keller, R.C., Killian, J.A., de Kruijff, B. 1992. Anionic phospholipids are essential for the alpha-elix formation of the signal peptide of prePhoE upon interaction with phospholipid vesicles. *Biochemistry* **31**:1672–1677

Kim, J., Kim, H. 1986. Fusion of phospholipid vesicles induced by α-Lactalbumin at acidic pH. *Biochemistry* **25**:7867–7874

de Kroon, A.I., Timmermans, J.W., Killian, J.A., de Kruijff, B. 1990. The pH dependence of headgroup and acyl chain structure and dynamics of phosphatidylserine, studied by ^{2}H-NMR. *Chem. Phys. Lip.* **54**:33–42

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685

Leckband, D.E., Helm, C.A., Israelachvili, J. 1993. Role of calcium in the adhesion and fusion of bilayers. *Biochemistry* **32**:1127–1140

Martin, I., Defrise-Quertain, F., Decroly, E., Vandenbranden, M., Brasseur, R., Ruysschaert, J.M. 1993. Orientation and structure of the NH_2-terminal HIV-1 gp41 peptide in fused and aggregated liposomes. *Biochim. Biophys. Acta* **1145**:124–133

Meers, P., Bentz, J., Alford, D., Nir, S., Papahadjopoulos, D., Hong, K. 1988. Sinexin enhances the aggregation rate but not the fusion rate of liposomes. *Biochemistry* **27**:4430–4439

Meers, P., Mealy, T., Pavlotsky, N., Tauber, A.I. 1992. Annexin I-mediated vesicular aggregation: mechanism and role in human neutrophils. *Biochemistry* **31**:6372–6382

Murata, M., Takahashi, S., Kagiwada, S., Suzuki, A., Ohnishi, S. 1992. pH-dependent membrane fusion and vesiculation of phospholipid large unilamellar vesicles induced by amphiphilic anionic and cationic peptides. *Biochemistry* **31**:1986–1992

Ohki, S. 1982. A mechanism of divalent ion-induced phosphatidylserine membrane fusion. *Biochim. Biophys. Acta* **689**:1–11

Parish, C.R., Classon, B.J., Tsagaratos, J., Walker, I.D., Kirszbaum, L., McKenzie, I.F. 1986. Fractionation of detergent lysates of cells by ammonium sulfate-induced phase separation. *Anal. Biochem.* **156**:495–502

Petri, W.A., Jr., Wagner, R. 1979. Reconstitution into liposomes of the glycoprotein of vesicular stomatitis virus by detergent dialysis. *J. Biol. Chem.* **254**:4313–4316

Pedemonte, C.H., Sachs, G., Kaplan, J.H. 1990. An intrinsic membrane glycoprotein with cytosolically oriented N-linked sugars. *Proc. Natl. Acad. Sci. USA* **87**:9789–9793

Pistolesi, R., Corazzi, L., Arienti, G. 1992. Microsomal protein mediates a pH-dependent fusion of liposomes to rat brain microsomes. *Membrane Biochem.* **9**:253–261

Rosenberg, J., Düzgünes N., Kayalar, C. 1983. Comparison of two liposome fusion assays monitoring the intermixing of aqueous contents and of membrane components. *Biochim. Biophys. Acta* **735**:173–180

Seddon J.M. 1990. Structure of the inverted hexagonal (H_{II}) phase, and non-lamellar phase transition of lipids. *Biochim. Biophys. Acta* **1031**:1–69

Surewicz, W.K., Epand, R.M., Epand, R.F., Hallett, F.R., Moscarello, M.A. 1986. Modulation of myelin basic protein-induced aggregation and fusion of liposomes by cholesterol, aliphatic aldehydes and alkanes. *Biochim. Biophys. Acta* **863**:45–52

Terzaghi, A., Tettamanti, G., Masserini, M. 1993. Interaction of glycosphingolipids and glycoproteins: thermotropic properties of model membranes containing G_{M1} ganglioside and glycophorin. *Biochemistry* **32**:9722–9725

Turnois, H., Fabrie, C.H., Burger, K.N., Mandersloot, J., Hilgers, P., Van Dalen, H., de Gier, J., de Kruijff, B. 1990. Gramicidin A induced fusion of large unilamellar dioleoylphosphatidycholine vesicles and its relation to the induction of type II nonbilayer structures. *Biochemistry* **29**:8297–8307

Wilschut, J., Düzgünes, N., Fraley, R., Papahadjopoulus, D. 1980. Studies on the mechanism of membrane fusion: kinetics of calcium ion induced fusion of phosphatidylserine vesicles followed by a new assay for mixing of aqueous contents. *Biochemistry* **19**:6011–6021

Wunderli-Allenspach, H., Ott, S. 1990. Kinetics of fusion and lipid transfer between virus receptor containing liposomes and influenza virus as measured with Octadecylrhodamine B Chloride assay. *Biochemistry* **29**:1990–1997