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A Glycoprotein from Rat Liver Endoplasmic Reticulum Promotes Both Aggregation and Fusion of Liposomes at Acidic pH

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Abstract. Low-pH-induced fusion of liposomes with rat liver endoplasmic reticulum was evidenced. Fusion was inactivated by treatment of microsomes with trypsin or EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline), indicating the involvement of a protein. The protein was purified 555-fold by chromatographic steps. The identification and purification to homogeneity was obtained by electroelution from a slab gel, which gave a still active protein of about 50 kDa. The protein promoted the fusion of liposomes; laser light scattering showed an increase of mean radius of vesicles from 60 up to about 340 nm. Fusion was studied as mass action kinetics, describing the overall fusion as a two-step sequence of a second order aggregation followed by a first order fusion of liposomes. For phosphatidylcholine containing liposomes aggregation was not rate-limiting at pH 5.0 and fusion followed first order kinetics with a rate constant of $13 \cdot 10^{-3}$ sec^{-1}. For phosphatidylethanolamine/phosphatidic acid liposomes aggregation was rate-limiting; however, the overall fusion was first order process, suggesting that fusogenic protein influences both aggregation and fusion of liposomes. The protein binds to the lipid bilayer of liposomes, independently of pH, probably by a hydrophobic segment. Exposed carboxylic groups might be able to trigger pH-dependent aggregation and fusion. It is proposed that the protein inserted in the lipid bilayer bridges with an adjacent liposome forming a fused doublet. Since at endoplasmic reticulum level proton pumps are operating to generate a low-pH environment, the membrane bound fusogenic protein may be responsible for both aggregation and fusion of neighboring membranes and therefore could operate in the exchange of lipidic material between intracellular membranes.

Key words: Endoplasmic reticulum and fusogenic activity — Fusogenic protein — glycoprotein — Liver — Liposomes

Introduction

Fusion between membranes is an essential and widespread biological event controlled by different mechanisms, some aspects of which have already been elucidated. Exocytosis and fertilization require Ca^{+2} ions (Ekerdt, Dahl & Gratzl, 1981; Kim & Kim, 1986), whereas in other processes this ion is not involved. During endocytosis, plasma membrane internalizes and reaches the endosomes, where a fusion process occurs, triggered by an acidified medium (Dean, Jessup & Roberts, 1984). The alphavirus Semliki Forest enters its host cell through receptor-mediated endocytosis and subsequent fusion with acidic endosomes, operated by the envelope glycoprotein of the virus (Moesby et al., 1995).

Endoplasmic reticulum is a membranous system that belongs to the elements of the exo- and endocytic pathways (Mellman, Fuchs & Helenius, 1986). By means of specific transport vesicles and membrane fusion events, it is involved both in the inward and in the outward material exchange with other cellular components. A great deal of evidence supporting the concept of flow of membrane constituents from and to endoplasmic reticulum derive from studies of biosynthesis, processing, sorting and transport of membrane or secretory glycoproteins (Abeijon & Hirschberg, 1988; Moreau et al., 1991; Slomiany et al., 1992). Most of the enzymes responsible for lipid biosynthesis reside on the rough and smooth

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endoplasmic reticulum. Consequently, the cellular segregation of these lipid-synthesizing activities needs an efficient interorganelle translocation to maintain the lipid composition of all cellular membranes (Kobayashi & Pagano, 1989). An intense lipid trafficking from and to the endoplasmic reticulum is indeed observed (Voelker, 1991).

Several hypotheses have been proposed to explain how membrane structures acquire lipid components (Vance, 1990). Recently, studies demonstrating that membrane contact and fusion points can be responsible for mutual phospholipid exchange between membranes have been published (Shiao, Lupo, & Vance, 1995; Camici & Corazzi, 1995).

In this paper we report that a glycoprotein purified from rat liver endoplasmic reticulum is able to trigger a pH-dependent fusion of liposomes. The fusion process has been studied in terms of mass action kinetics. This model describes the overall reaction as a two-step sequence, consisting of a second-order process of liposome aggregation followed by a first-order fusion reaction (Nir, Bentz & Wilschut, 1980). Results obtained by studying the aggregation and fusion of liposomes of different lipid composition indicate that the fusogenic protein promotes both the aggregation and the fusion stages.

Materials and Methods

Materials

HEPES, Thesit, n-octylglucoside and MES were produced by Boehringer-Biochemie (Mannheim, Germany). Sephadex G-50 and Con A-Sepharose were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). DEAE-Biogel A, Biogel HTP Hydroxyapatite and SDS-polyacrylamide gel electrophoresis reagents were from Bio-Rad (Richmond, CA). Pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) and $TbCl_3 \cdot 6H_2O$ were purchased from Aldrich Chemie (Steinheim, FRG). Octadecyl rhodamine B chloride (R_{18}) was from Molecular Probes (Eugene, OR). Lectin from *Triticum vulgaris* and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) were from Sigma Chemical (St. Louis, MO). Phosphatidic acid from yolk lecithin and bovine brain phosphatidylethanolamine, purchased from Sigma Chemical, were purified in our laboratory, prior to use, as described in the Analytical Procedures. α-Methylmannopyranoside was from Fluka Chemie AG Buchs (Switzerland).

Preparation of Subcellular Fractions

Livers obtained from male Wistar rats (approximately 200 g) were homogenized in 0.25 M sucrose plus 5 mM HEPES, pH 7.4 (S/H buffer). The homogenate was centrifuged twice at 1,500 × *g* for 10 min and the pellets were discarded. The supernatant was centrifuged at 8,000 × *g* for 20 min to eliminate crude mitochondrial fraction by pelleting. Lysosomes and other particles were removed by pelleting at 20,000 × *g* for 30 min. The resulting supernatant was centrifuged at 105,000 × *g* for 60 min in a Beckman 60 Ti-rotor. Sedimented microsomes were resuspended in the correct amount of S/H buffer. In some instances the whole microsomes were separated into smooth and rough fractions by centrifugation on sucrose density gradient (Steer, Klausner & Blumenthal, 1982).

Characterization of whole microsomes and of smooth and rough fractions was performed by testing the activity of the proper marker enzymes. The specific activities of cytochrome c oxidase, mitochondrial marker enzyme and of UDP-N-acetylglucosamine-1-phosphotransferase, Golgi complex marker enzyme (Rusiñol et al. 1994), were 4–6% of the specific activities found in the crude mitochondrial fraction. Arylsulfatase A activity, marker for lysosomes (Percy et al., 1983), was negligible if compared to that of lysosomal fraction. Instead, the specific activity of NADPH cytochrome c reductase, marker enzyme for endoplasmic reticulum (Rusiñol et al., 1994), was enriched 10–12 times compared to that found in the homogenate. The RNA content (Karsten & Wollenberger, 1972) of rough membranes (200 ± 30 μg/mg protein) was about ten times higher than that of smooth membranes.

Insertion of R_{18} Into Microsomal Membranes

Microsomes were labeled with R_{18} (Mcr-R_{18}) by injection of 10 μl of an ethanolic solution of R_{18} (25 μg) into 1 ml of S/H solution containing the vesicles (1 mg protein, about 1.5 mM total lipid) (Camici & Corazzi, 1997). About 70% of fluorophore was incorporated into microsomes, this implying a surface density of ≤0.02 mol of probe/mol of microsomal lipid. At controlled temperature (20°C), the fluorescence quenching was proportional to the concentration of the fluorophore in the lipid phase. This permitted the calculation of fusion extent from fluorescence dequenching.

Special Treatment of Microsomes

In some instances microsomes were treated with special reagents before loading with R_{18}. In the treatment with trypsin, microsomes were suspended in S/H buffer at a concentration of 4-mg protein/ml and incubated with or without (controls) 0.2 mg/ml of trypsin for 10 min at 30°C. The reaction was stopped by chilling the samples to 4°C. Microsomes were subsequently diluted (1:10, by vol.) with S/H buffer and harvested by centrifugation at 105,000 × *g* for 60 min.

To treat microsomes with EEDQ, 5 mg of microsomal protein in 0.5 ml of 0.25-M sucrose and 10-mM MES (pH 5.5) were added with 50 μl of 5-mM EEDQ in ethanol. Ethanol alone was added to controls. Samples were incubated at 37°C for 60 min and nonreacted EEDQ was eliminated by filtration through a Sephadex G-50 column (1 × 30 cm) and eluted with S/H buffer. Microsomes were also treated with 1-M NaCl or 3-M guanidine hydrochloride or with 4-M urea using a procedure described in Results.

Preparation of Liposomes

If not otherwise specified, liposomes prepared with phospholipids extracted from rat liver microsomes were used throughout this work. Phospholipids were resuspended in a proper medium (about 1.5 μmol lipid P/ml). The suspension was sonicated with a MSE tip sonicator to clearing. Metal particles from the sonicator tip and multilamellar liposomes were removed from the preparation by centrifugation at 100,000 × *g* for 20 min. Alternatively, liposomes were prepared by detergent removal as described (Rakowska, Zborowski & Corazzi, 1994). Liposomes used to test the fusogenic activity of microsomes with the R_{18} method or used in the laser light scattering measurements, were prepared in S/H buffer. Tb^{+3}- and DPA-loaded liposomes were prepared in (mM): 2 HEPES and 2 L-histidine (pH 7.4) containing (i) 15 $TbCl_3$ and 150 sodium citrate, (ii) 150 dipicolinic acid (sodium salt) (Wilschut

et al., 1980). Unencapsulated material was eliminated by gel filtration on Sephadex G-50 column, as described (Corazzi et al., 1989); the removal was complete, as demonstrated by the absence of the fluorescent peak at 545 nm after mixing DPA-liposomes with free Tb^{+3} (0.75 μM) or Tb^{+3}-liposomes with free DPA (5 μM). The same procedure showed that the leakage of the probes from the vesicles was negligible for at least 120 min. The composition of liposomes was the following (molar ratio): phosphatidylcholine, 49%; phosphatidylethanolamine, 29%; phosphatidylserine, 6%; phosphatidylinositol + sphingomyelin, 11%; others, 5%. In some experiments liposomes were prepared from pure PE and PA, using the described procedure.

Purification of the Fusogenic Protein

Microsomes (100 mg protein, 15 ml in S/H buffer) were mixed with 2% Thesit solution (15 ml in S/H buffer) and the resulting solution was stirred for 10 min at 4°C. Unsolubilized material was pelleted by centrifugation (105,000 × *g* for 1 hr) and supernatant treated with ammonium sulfate up to 30% saturation, in order to obtain phase separation. After centrifugation (105,000 × *g* for 1 hr), the upper hydrophobic phase was eliminated and the lower phase, containing the fusogenic activity, extensively dialyzed against S/H buffer. After dialysis the material (49 mg protein) was chromatographed through DEAE Biogel A column (1 × 10 cm) pre-equilibrated in S/H buffer. Elution was performed with three discontinuous steps of S/H buffer containing 0.05, 0.2 and 1-M NaCl. The peak eluted with 0.2-M NaCl was treated with ammonium sulfate up to 50% saturation and proteins, precipitated by centrifugation (105,000 × *g* for 1 hr), were resuspended in 5-mM HEPES and 1-M NaCl (pH 7.4) (Na/H buffer) and dialyzed against the same solution. Proteins (6.3 mg, 2 ml) were passed through a Con A-Sepharose 4B column (1 × 4 cm) pre-equilibrated with 0.1-M acetate buffer (pH 6.0) containing 1-M NaCl, 1-mM $MgCl_2$ and 1-mM $MnCl_2$, followed by Na/H buffer. After washing with Na/H buffer, the retained fusogenic protein was desorbed by eluting with Na/H buffer containing 1% Thesit and 0.5 M α-methylmannopyranoside. The detergent was removed by phase separation as described above. The partially purified fusogenic protein (0.7 mg) was applied to a Biogel HTP column (1 × 4 cm) pre-equilibrated in 0.2-M sucrose and 1-mM NaCl solution (not buffered). Elution was performed with discontinuous steps of 0.2-M sucrose containing respectively 0.01, 0.10, 0.3 and 0.50-M sodium phosphate (pH 7.4). Fusogenic activity was found distributed in the 0.1-M phosphate eluent. After dialysis against Na/H buffer the material was concentrated using Centriplus-10 (Amicon) and proteins (about 0.4 mg) recovered in 0.5 ml were passed through a lectin from *Triticum vulgaris* on 6% agarose column (0.5 × 3 cm) pre-equilibrated with Na/H buffer. Elution was performed with: (i) Na/H buffer, (ii) Na/H buffer containing 0.1-M N-acetylglucosamine. Fusogenic material, eluted with the second buffered eluent, was dialyzed against S/H solution and concentrated as above. SDS-polyacrylamide slab gel electrophoresis was then performed, omitting the starting boiling step of proteins. Protein bands were localized through a reference lane on the gel stained with the silver nitrate method. Proteins were electroeluted by the Bio-Rad electro eluter (model 422) using 0.2 M glycine (pH 8.8) as running buffer. Recovered proteins were concentrated with Microcon-10 (Amicon) by repeated additions of S/H buffer and then tested for their fusogenic activity.

Fusion Assay

R_{18} (Hoekstra et al., 1984) or Tb/DPA (Wilschut et al., 1980) assays were used to monitor the fusion process. With either method fluorescence was measured using a Perkin-Elmer MPF-3 spectrofluorometer equipped with a cutoff filter (UV31) to eliminate contribution to signal due to light scattering.

Fusion of liposomes to Mcr-R_{18} was monitored at 560 and 580 nm excitation and emission wavelengths, respectively. Windows were set at 4 nm. Fusion was initiated by injecting 100 μl of liposomes (50 nmol lipid P) into a cuvette containing (in mM): 100 sucrose, 50 NaCl, 20 MES (at carefully controlled pH values) and Mcr-R_{18} (about 30 μg protein) in a final volume of 2 ml. The minimal initial fluorescence of Mcr-R_{18} before the addition of liposomes was set to 0%. The fluorescence in the presence of 0.03% Thesit was taken as 100%.

The Tb/DPA fusion assay was used to test the activity of the fusogenic protein during the purification procedure and during the characterization studies. Even though the fusogenic protein was released from microsomes with detergent, it was sufficiently water soluble. Therefore, after each purification step, the fusogenic activity was tested by adding proteins as an aqueous solution to a mixture of TB^{+3}- and DPA-liposomes and fusion process monitored as described below. The fusion assay was carried out in a cuvette containing 2 ml of 100-mM sucrose, 50-mM NaCl, 20-mM MES (pH 5.0), Tb^{+3}- and DPA-liposomes. Excitation wavelength was set at 276 nm and fluorescence emission detected at 545 nm after insertion of the UV31 filter. Windows were set at 10 nm for excitation and 6 nm for emission. Fusion was initiated by the addition of an aliquot of the protein and the fusogenic activity was expressed as described in the figure legends. The value of maximal fluorescence of Tb^{+3}- and DPA-loaded liposomes, determined in the presence of 0.03% Thesit, allowed comparison of different liposomal preparations used in the experiments. The overall fusion was described by the forward and reverse aggregation rate constants between two single vesicles (V_1) to form a dimer aggregate (V_2) that will fuse to form a fusion product (F_2) (Walter & Siegel, 1993). Vesicle aggregation was followed by changes in 90° light scattering at 400 nm.

Density Gradient Centrifugation of Fusion Products

DPA-liposomes (750 nmol lipid P, 0.5 ml) were mixed with 0.5 ml of either (in mM): 100 sucrose, 50 NaCl plus 100 MES buffer (pH 5.0) or 100 sucrose, 50 NaCl plus 20 HEPES (pH 7.4). Fusogenic protein (10 μg) was added to each sample and fusion process was carried out for 10 min. Fusion products were passed through a Sephadex G-50 column (1 × 20 cm) equilibrated in S/H buffer, and the material eluted in the void volume was collected. Samples were layered on top of continuous glycerol gradients (5 to 50%, w/v) buffered with 5-mM HEPES (pH 7.4). Centrifugation was performed at 250,000 × *g* in a Beckman VTI65 vertical rotor for 4 hr at 4°C. In a control experiment a sample of fusogenic protein (10 μg) was centrifuged in the same experimental conditions. After centrifugation, 0.5-ml fractions were collected from a hole made at the bottom of the tubes. The fusogenic activity of recovered DPA-liposomes was tested adding Tb^{+3}-liposomes at acidic pH.

Affinity Chromatography of Fusion Products

Liposomes (750 nmol lipid P in 0.5 ml of 0.1-M NaCl and 5-mM HEPES, pH 7.4) were mixed with 0.5 ml of 0.1-M NaCl and 100-mM MES (pH 5.0). Fusogenic protein (10 μg) was then added and fusion process was carried out for 10 min. In a parallel experiment the procedure was performed omitting the fusogenic protein. The material was passed through a concanavalin A column (1 × 5 cm) equilibrated with 20-mM HEPES and 0.1-M NaCl (pH 7.4). After several washings, the bound liposomes were eluted with the same buffer containing 0.5-M

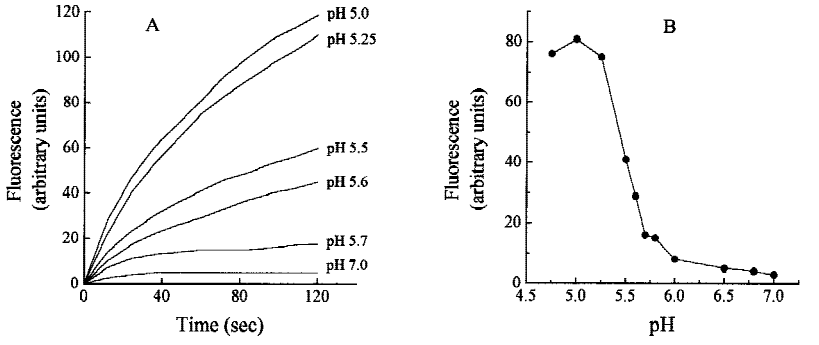

Fig. 1. Influence of pH on the fusion of liposomes to microsomes. (*A*) Mcr-R_{18} (30-μg protein) and liposomes (50-nmol lipid P), were added to 2 ml of 100-mM sucrose, 50-mM NaCl solution buffered with 20-mM MES in the 4.75–7.0 pH range. Fusion was initiated by injection of the liposomes, and the increase of R_{18} fluorescence was measured. (*B*) pH-dependence of the initial fusion rate. Data are expressed as fluorescence elicited during the first 30 sec.

α-methylmannopyranoside and 4 mg/ml of octylglucoside. The material eluted from the column was then analyzed for the lipid P content.

Laser Light Scattering

A photon correlation measurement of the light scattered at a desired angle may give the translational diffusion coefficient *D*. For spherical structures, *D* is related to the effective hydrodynamic radius of translation R_H through the Stokes-Einstein relation: $D = KT/6\pi\eta R_H$, where *K* is the Boltzmann constant, *T* is the absolute temperature and η is the viscosity of the solvent. Therefore measurement of *D* at a known temperature yields the value of R_H.

In our experiments all buffers were filtered through 0.22-μm pore Millipore filters prior to use. The concentration of liposomes was 50-μM lipid phosphorus in a buffer (2 ml) composed of (mM): 100 sucrose, 50 NaCl and 20 HEPES (pH 7.4) or 20 MES (pH 5.0). Fusogenic protein (3 μg) was added for fusion. The laser light source was a NEC GLG-5730 He-Ne laser (632.8 nm) producing 50 mW of optical power. The scattered photo pulses were amplified by a BI-9836 photomultiplier and then passed to a BI-9000 Digital correlator. Temperature, kept at 20°C, was controlled by a digital thermometer positioned in the light scattering cell holder. Data analysis was performed using both a double and a multiple exponential fitting of the autocorrelation function, to obtain the diameter populations of the liposomes.

Analytical Procedures

SDS-polyacrylamide slab gel electrophoresis was carried out as described (Laemmli, 1970). The gel was run using the Bio-Rad Mini Protean apparatus and stained with the silver nitrate method (Giulian, Moss & Greaser, 1983). Total lipids were extracted from liver microsomes (Folch, Lees & Sloane Stanley, 1957). Neutral lipids, glycolipids and proteins were removed by column chromatography (Rouser, Kritchevsky & Yamamoto, 1967). Phospholipid composition was determined after separation of each lipid class by two-dimensional TLC (Camici & Corazzi, 1995). PA and PE, purified by two-dimensional TLC, were revealed by staining with dichlorofluorescein, scraped off the plate and eluted with chloroform-methanol-acetic acid-water (50: 39:1:10, v/v/v/v). Samples were freed from dichlorofluorescein by washing three times with 4N ammonia and three times with methanol-water (1:1, v/v). Protein was quantified as described (Bradford, 1976); phospholipid phosphorus was assayed after digestion with 70% perchloric acid (Barlett, 1959).

Abbreviations

HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; MES, 2-(N-morpholino)-ethanesulfonic acid; S/H buffer, sucrose-HEPES buffer (0.25-M sucrose + 5-mM HEPES, pH 7.4); Na/H buffer, 1-M NaCl + 5-mM HEPES, pH 7.4; Thesit, dodecylpoly (ethylenglycolether)$_9$; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; R_{18}, octadecylrhodamine B chloride.

Results

Fusion of Liposomes to Microsomes

Incubation of Mcr-R_{18} with liposomes resulted in fluorescence dequenching, observed as an increase in fluorescence over a time course of a few minutes. Dequenching increases upon decreasing the pH of incubation medium, indicating that microsomal membranes can acquire liposomes depending on the pH (Fig. 1*A*). At pH 7.0 no fluorescence increase was found up to 2 min. Lowering the pH a fast and pronounced fluorescence increase was observed. Zero and 100% fluorescence points were determined preliminarily in each case. Moreover, all the reported kinetics are comparable since the fluorescence intensity of R_{18} is independent of pH (Arbuzova et al., 1994). As shown in Fig. 1*B*, a sharp increase of the initial rate of the fluorescence development was observed at pH values lower than 5.75, with a maximum at pH 5.0. The same results were obtained when experiments were performed with liposomes prepared by detergent removal (*not shown*). Since the R_{18} dequenching occurs only after the addition of liposomes, a pH-sensitive membrane protein should be involved in the fusion process. A demonstration of this statement was further inferred by the results of two experimental approaches here described. In the first, microsomes were reacted with a low concentration of trypsin, to act upon proteins localized on the external surface of the membranes. In the second, microsomes were reacted with EEDQ, a derivatizing compound for carboxylic groups of proteins (Pougeois, Satre & Vignais, 1978). Trypsin- and EEDQ-treated microsomes were then labeled with R_{18} and tested for their fusogenic activity. Results indicated that fusogenic activity of microsomes was lost in both cases.

Table. Steps of fusogenic protein purification

	Protein (mg)	Activity (fluorescence units)	Specific activity	Purification (fold)	Yield (%)
Microsomes	100	318,913	3,189	1	100
$(NH_4)_2SO_4$	48.9	287,020	5,869	2	90
DEAE-biogel A	6.3	109,562	17,390	15.9	34.3
Con-A	0.7	31,130	44,471	143	9.8
Biogel-HTP	0.38	22,215	58,460	263	7.0
Triticum vulgaris	0.18	20,000	111,111	555	6.3

The fusogenic activity is expressed in arbitrary fluorescence units measured 2 min after mixing of Tb^{+3}- and DPA-loaded liposomes (*see* Materials and Methods). Estimated fluorescence intensity was proportional to the protein concentration.

Removal of Peripheral Proteins

To determine whether the fusogenic factor is an integral or peripheral membrane protein, microsomal suspensions (1-mg protein, 2 ml in S/H buffer) were incubated at 4°C for 20 min in the presence of 1-M NaCl or 3-M guanidine hydrochloride or 4-M urea. In controls, perturbing agents were omitted. After the treatment, samples were centrifuged at 105,000 × *g* for 60 min and the resulting pellets were resuspended in 2 ml of S/H buffer. Samples were further centrifuged twice and final microsomal pellets resuspended in 0.5 ml of S/H buffer. Microsomes were then loaded with R_{18} and fusogenic activity was tested at pH 5.0. The fusogenic activity was preserved in each treated sample, indicating that the fusogenic factor is tightly bound to the membrane. It is worth noting that the performed treatments dissociate peripheral proteins, as demonstrated by SDS-PAGE analysis of supernatant (*not shown*).

Purification of the Fusogenic Protein

Fusogenic protein was purified from rat liver endoplasmic reticulum by a modification of the described procedure (Rakowska et al., 1994). The Table reports the results of the purification steps. The fusogenic activity was determined after each purification step and was expressed in arbitrary units of fluorescence intensity after mixing of Tb^{+3}- and DPA-loaded liposomes in the presence of the fusogenic protein. The relative rates of fusion catalyzed by the protein fractions that were discarded in relation to the purified protein were negligible. The treatment of membranes with 1% Thesit solubilized 90% of proteins. Subsequent ammonium sulfate addition determined phase separation of lipids and detergent in the upper phase. At the same time, 48.9% protein and 90% fusogenic activity were recovered in the lower phase. Fusogenic protein was bound to DEAE-Biogel A and eluted at 0.2-M NaCl in buffered solution. This chromatographic step increased three times the specific activity of the protein and resulted in a good purification factor. Purification was continued with Con-A Sepharose, which retained the fusogenic activity, indicating its glycoproteic nature. The next purification step utilized hydroxyapatite column. Displacement of bound fusogenic protein occurred at 100-mM phosphate buffer. Proteins eluted with 10, 300 and 500-mM phosphate, did not possess fusogenic activity. The further chromatographic step performed with *Triticum vulgaris* retained about 50% glycoproteins. Fusogenic protein could be eluted only after passage of N-acetylglucosamine. At this stage, SDS-PAGE showed several protein bands. The identification of the fusogenic protein was possible since electroelution of the bands from the gel yielded a still active fusogenic protein with an approximate molecular weight of 50 kDa (Fig. 2). The purification procedure was carried out from either rough or smooth endoplasmic reticulum. Fusogenic protein was found in both membrane preparations, with no substantial differences in the recovery of the fusogenic activity. The pure and active protein eluted from the gel was utilized for all reported experiments.

Photon Correlation Spectroscopic Studies

The effect of the fusogenic protein on the average size and size distribution of the vesicles has been determined by laser light scattering, as described in Methods. At pH 7.4 the calculated mean radius was 60 ± 15 nm. The lowering of the pH from 7.4 to 5.0, resulted in the modification of size distribution: 126 ± 36 nm. Further, at pH 5.0, the addition of the fusogenic protein to a liposome suspension determined a noticeable change of vesicle structures. Indeed, after fusion, calculated mean radius of about 50% vesicle population was increased up to 338 ± 80 nm.

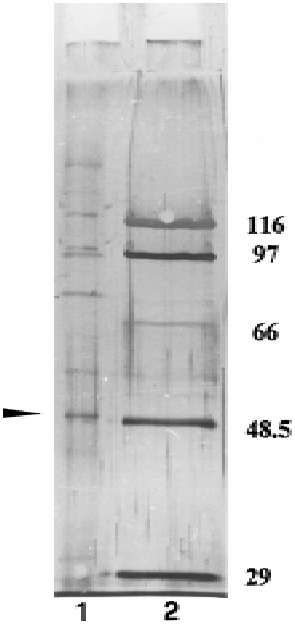

Fig. 2. SDS-PAGE. Lane 1, proteins eluted from *Triticum vulgaris* column; lane 2, molecular mass markers (β-galactosidase, 116,000; phosphorylase b, 97,000; serum albumin, 66,000; fumarase, 48,500; carbonic anhydrase, 29,000). The gel (8% acrylamide) was stained using the silver nitrate method. The arrow indicates the band possessing fusogenic activity after electroelution.

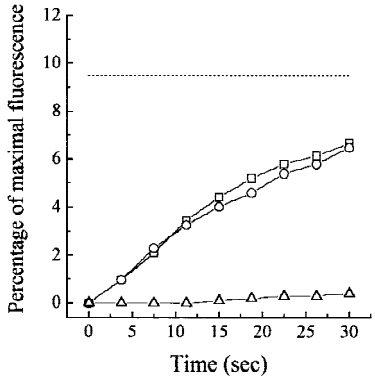

Fig. 3. Fusion of liposomes induced by the fusogenic protein: evaluation of leakage. Tb^{+3}- and DPA-liposomes (64-nmol lipid P each) were mixed in a cuvette containing 2 ml of 100 mM sucrose, 50-mM NaCl and 20-mM MES (pH 5.0). Fusogenic protein (3 μg) was added in absence (□-□) or in presence (○-○) of free DPA (10 μM). The fluorescence of the Tb/DPA complex formed upon fusion of liposomes was monitored and reported as % of maximal fluorescence emitted in the presence of 0.03% Thesit. Elicited fluorescence expected if fusion had occurred with a leaky process is also indicated (dotted line). Control sample for measuring the spontaneous leakage of liposomes was made by mixing Tb^{+3}-liposomes and free DPA (△-△).

Fusion Process is Nonleaky

To demonstrate that Tb^{+3}- and DPA-liposomes fusion triggered by the fusogenic protein is a nonleaky process, the following experiment was performed. Equal amounts of Tb^{+3}- and DPA-liposomes (64 nmol lipid P each) were mixed at pH 5.0 in the presence of the fusogenic protein (3 μg). Kinetics of fluorescence emission of Tb/DPA complex formation was followed in absence or in presence of free DPA in the incubation mixture. At 10-μM free DPA, developed fluorescence intensity was not increased, contrary to what we expected if a leaky process in Tb-Tb liposomes fusion had occurred (Fig. 3). From fluorescence data and considering negligible multiple fusion events, it is calculated that 13% of liposomes (16.6 nmol lipid) are fused within 0.5 min (Nir et al., 1980). Of this percentage, 1/4 (about 4-nmol lipid) must be ascribed to the fusion of Tb-Tb liposomes, which should be productive in terms of fluorescence only if the process is leaky and free DPA is present in the medium. In this case one should expect an increase in elicited fluorescence (Fig. 3, dashed line), since 4-nmol Tb-liposomes develop about 3% of total fluorescence.

Vesicle Concentration Dependence of Fusion

The role of the fusogenic protein in the two steps of the overall fusion process, i.e., aggregation and fusion, has been examined. The aggregation and fusion rates are susceptible to factors such as phospholipid composition, concentration of liposomes and pH. We performed the phospholipid concentration dependence of lipid mixing, to study the role of the fusogenic protein in a condition for which aggregation or fusion *per se* are the rate-limiting steps.

In the first set of experiments, we have used liposomes prepared with total phospholipids from microsomal membranes. They aggregate spontaneously at a pH lower than 7.0, as pointed out by measuring the increase of light scattering intensity at 400 nm (*result not shown*). Hence, for this kind of liposomes, at pH 5.0 aggregation is not a rate-limiting step and, consequently, the fusogenic protein should act at the fusion level step. To confirm this hypothesis the *log* of the initial rate of Tb fluorescence increase was plotted against the *log* of the vesicle concentration (Fig. 4). The plotting of the data at the lower vesicle concentrations yielded a straight line with a slope of 1.1. Above 10 μM vesicle concentration the relation was still linear, with a slope of 0.41. It is concluded that kinetics of fusion of this kind of liposomes are controlled by the fusion rate constant, since a slope of about 1 or lower indicates a first order of liposome fusion, i.e., aggregation step does not determine the overall fusion rate. Other experiments performed at very low liposome concentrations showed that the rate of fusion increased linearly as a function of fusogenic protein concentration, in the range of protein concentrations used (Fig. 5). This result further indicates that the overall fusion process is not limited by the aggregation step.

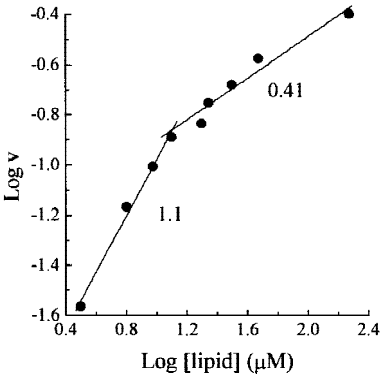

Fig. 4. Dependence of initial rate of fusion on liposome concentration. Different concentrations of Tb^{+3}- and DPA-liposomes (1:1, molar ratio) were mixed in a cuvette containing 2 ml of 100-mM sucrose, 50-mM NaCl and 20-mM MES (pH 5.0), in the presence of the fusogenic protein (3 μg). Initial rates of fluorescence increase, expressed as a percentage of maximal fluorescence per min, were determined and corrected for the actual lipid concentration, relative to 100 μM. Logarithm of initial rate of fusion was then plotted *vs.* logarithm of vesicle concentration. The numbers under the curve indicate the slope of each portion.

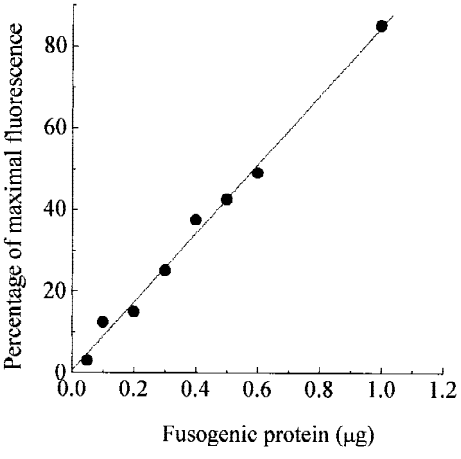

Fig. 5. Rate of fusion of liposomes as a function of fusogenic protein concentration. Tb^{+3}- and DPA-liposomes (6-nmol lipid each) were mixed in a cuvette containing 2 ml of 100 mM sucrose, 50-mM NaCl and 20-mM MES (pH 5.0) and fusion was initiated by addition of the indicated amount of protein. Initial fusion rates, expressed as a percentage of maximal elicited fluorescence per min, are reported.

If in the process $V_1 + V_1 \rightarrow V_2 \rightarrow F_2$ aggregation is not a limiting step, the first order integrated equation is $K = 2.3\ log(a/a\text{-}x)/t$, where K is the fusion rate constant, t is the time in sec, a is the initial vesicle concentration of liposomes and x the concentration of fused vesicles. The molar concentration of the vesicles is calculated from the lipid concentration using a number of 80,000 lipid molecules per vesicle. During the initial stage of fusion, the fluorescence is primarily due to fused doublets, since the higher fusion orders can be negligible. Hence, if all the

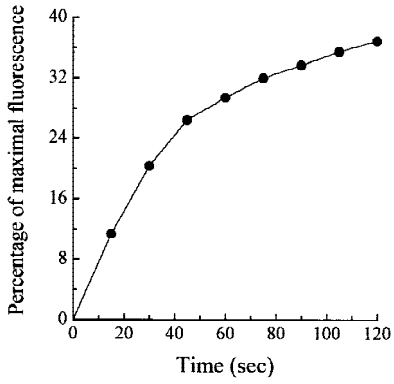

Fig. 6. First order kinetics of liposome fusion. Tb^{+3}- and DPA-liposomes (12.6-nmol lipid P each) were mixed in a cuvette containing 2 ml of 100 mM sucrose, 50-mM NaCl and 20-mM MES (pH 5.0). Fusion was initiated by addition of fusogenic protein (1 μg). Tb/DPA fluorescence increase, expressed as percentage of maximal fluorescence obtained in the presence of 0.03% Thesit, is reported.

vesicles fuse to doublets, the higher obtainable fluorescence will be 50% of the maximal, since only one-half of these doublets would contain both Tb and DPA. From the kinetics reported in Fig. 6 the value determined for K is $13 \cdot 10^{-3} \pm 1.5 \cdot 10^{-3}$ sec^{-1}. To correlate the rate constant to the time course of the fusion event, we assume that all vesicles aggregate into dimers, V_2, which produce the fused doublets, F_2. Therefore $d[F_2]/dt = K[V_2]$. For the initial reaction time the equation can be solved into $[F_2] \approx [V_2^{\circ}]Kt$ (Nir, Wilschut & Bentz, 1982) where $[V_2^{\circ}]$ is the initial concentration of dimers. According to this relationship, it is possible to determine that the time needed for the fusion of 1% of vesicles is about 0.8 sec.

In the second set of experiments we have used PE/PA (1:1) liposomes. The light scattering of PE/PA liposomes did not increase upon decreasing the pH of the medium from 7.4 to 5.0, indicating that H^+ fails to promote liposome aggregation. Therefore, since the aggregation process is second order in vesicle concentration, one should expect that the overall fusion process is controlled by the aggregation step. On the contrary, if the *log* of the initial rate of Tb fluorescence increase is plotted against the *log* of the vesicle concentration, a straight line with a slope less than 1 is obtained (*not shown*). Further, the fusion rate constant, K, determined from the initial rate of liposome fusion fits the first order integrated equation and, for any vesicle concentration, is $15 \cdot 10^{-3} \pm 1.2 \cdot 10^{-3}$ sec^{-1}. This result was confirmed when fusion kinetics of PE/PA liposomes were performed in a wide range of phospholipid concentrations, keeping constant the phospholipid/fusogenic protein ratio. From 5 to 50-μM phospholipid concentration, the percentage of maximal elicited fluorescence, at each time, was comparable for any vesicle concentration (Fig. 7). This result indicates that also in the very diluted vesicle solutions aggregation is not a limiting step.

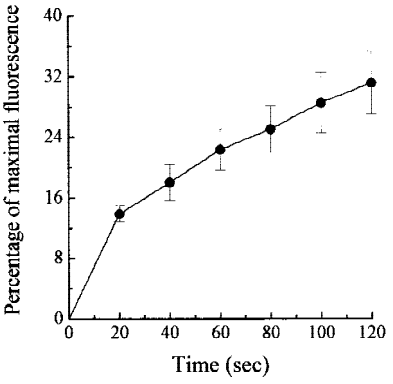

Fig. 7. Kinetics of PE/PA liposome fusion. Tb^{+3}- and DPA-liposomes (20, 40, 60 or 100 nmol lipid P each) prepared with pure PE/PA (1:1) were mixed in a cuvette containing 2 ml of 100 mM sucrose, 50-mM NaCl and 20-mM MES (pH 5.0). Fusion was initiated by addition of fusogenic protein. For all samples protein/phospholipid ratio was kept constant and was 0.03 μg protein/nmol lipid P. At fixed times, Tb/DPA fluorescence increase, expressed as percentage of maximal fluorescence obtained in the presence of 0.03% Thesit, was determined for each kinetics. Reported data are means ± SD of kinetics performed at four different phospholipid concentrations.

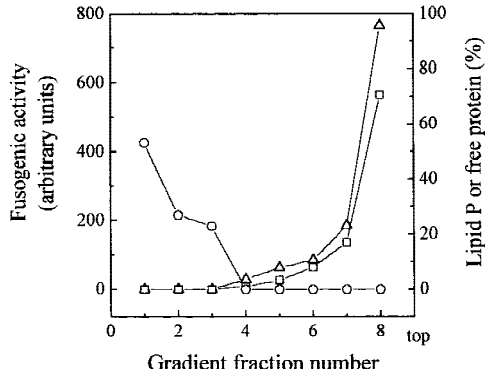

Fig. 8. Interaction of the fusogenic protein with liposomes: centrifugation of liposomes on glycerol density gradient. DPA-liposomes (750 nmol lipid P) were mixed with fusogenic protein (10 μg) in a buffered solution (pH 5.0) (*see* Materials and Methods). After 10 min liposomes were passed through a Sephadex G-50 column and the material eluted in the void volume (fusion product) was centrifuged on a discontinuous gradient of glycerol as described. Fractions collected after centrifugation were tested for fusogenic activity with Tb^{+3}-liposomes (△-△) and for lipid content (□-□). In the control experiment, a sample of fusogenic protein was centrifuged in the same experimental conditions (○-○).

Fusogenic Protein Interaction with Liposomes

The discovery that protein promotes both aggregation and fusion of liposomes prompted us to search information on the nature of protein-phospholipid interaction. Fusogenic protein was added to DPA-liposomes, at pH 5.0, and fusion was carried out for 10 min at 20°C. Thereafter the mixture was passed through a Sephadex G-50 column and eluted liposomes were harvested and centrifuged on glycerol density gradient. Figure 8 shows that all the fusogenic activity was found together with lipid in the upper part of the gradient. In a parallel experiment the fusogenic protein alone was recovered in the lower part of the gradient. When the same experiment was performed by mixing DPA-liposomes and fusogenic protein at pH 7.4, distribution of the fusogenic activity along the gradient was similar to that observed in the experiment at pH 5.0 (*not shown*). This result demonstrates that fusogenic protein may bind to the lipid bilayer of liposomes with a pH-independent process.

Additional experimentes were carried out taking advantage of the presence of the glucidic portion of the fusogenic protein. Liposomes mixed with the fusogenic protein at pH 5.0, were eluted through a Con A Sepharose column (Fig. 9). The majority strongly interact with Concanavalin A, their release being obtained only by eluting with α-methylmannopyranoside and detergent. On the contrary, in the absence of fusogenic protein, liposomes were largely eluted in the void volume of the column. These data could indicate that the protein spontaneously enters the lipid bilayer in such a way that its glucidic moiety faces the external side of the liposomes, making possible its interaction with Concanavalin A.

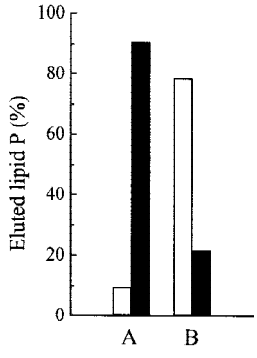

Fig. 9. Interaction of the fusogenic protein with liposomes: chromatography of liposomes through a Con-A Sepharose column. (*A*) 10 μg of fusogenic protein were added to liposomes (750-nmol lipid P) in a buffered solution (pH 5.0) (*see* Materials and Methods). After 10 min the sample was passed through a Con-A Sepharose column. (*B*) Control liposomes without fusogenic protein were treated as in (*A*). Unbound material (empty bars) was eluted with 0.1-M NaCl and 20-mM HEPES (pH 7.4). Bound material (filled bars) was eluted with the same buffer containing 0.5-M α-methylmannopyranoside and 4 mg/ml of octyl-β-D-glucopyranoside. Data are expressed as percentage of total loaded P lipid.

Discussion

The purification and some properties of a fusogenic protein associated with the endoplasmic reticulum are de-

scribed in this paper. The evaluation of biochemical markers suggests that contamination of microsomes with other subcellular fractions is low in our preparations. Coated vesicles, containing clathrin, a protein possessing fusogenic activity at acidic pH (Blumenthal, Henkart & Steer, 1983) were excluded from microsomal preparation, as checked by performing the subfractionation of microsomes (Steer et al., 1982).

In this work the fluorescence dequenching of the R_{18} probe inserted into microsomal membranes demonstrated that microsomes possess a pH-dependent fusion activity toward liposomes (Fig. 1). The critical importance of acidic pH values for the efficient membrane fusion can be related to the protonation level of proteins which promotes hydrophobic interactions between microsomes and liposomes. This fact is in agreement with the finding that maximal developed fusogenic activity is in the 5.0–5.75 pH range (Fig. 1*B*), i.e., below the pKa of residual carboxylic groups of proteins (Pougeois et al., 1978; Harb et al., 1986). Therefore, protein(s) tightly associated with the endoplasmic reticulum could be involved in the fusion event. This is also evidenced from the observation that treatment of membranes with trypsin resulted in an inhibition of approximately 90% of the fusogenic activity. Fluorescence dequenching of R_{18} was observed in both smooth and rough endoplasmic reticulum, indicating that the fusogenic protein is distributed in the whole reticulum. Many reports indicate the presence of membrane-bound fusogenic proteins in the cells. A protein exposed on the external side of the intestinal brush-border membrane captures liposomes (Thurnhofer, Lipka & Hauser, 1991). Evidence is also reported suggesting that Golgi integral membrane proteins are involved in fusion (Kagiwada et al., 1993). The involvement of proteins in the fusion of reticulocyte endocytic vesicles with liposomes has been reported (Vidal & Hoekstra, 1995). However, purification and characterization of such membrane-bound fusogenic proteins have not been described yet.

In our laboratory the fusogenic protein has been detected by electroelution from the gel, after several chromatographic steps for its purification (Table). The most noticeable characteristic of the protein is its glycoproteic nature, as evidenced by its strong interaction with Con A Sepharose column. Although the composition of the glucidic moiety has not been determined, the specific interaction of the protein with lectin from *Triticum vulgaris* should indicate the presence of N-acetylglucosamine dimer or trimer structures (Gougos & Letarte, 1988). The fusogenic protein possesses many carboxylic residues, as evidenced by the interaction with DEAE and HTP columns. The involvement of these residues in the fusion event was demonstrated by the finding that after derivatization of microsomal membrane proteins with EEDQ the fusogenic activity was lost. The ability to promote fusion of the purified protein must be specific in microsomes, as many other glycoproteins removed during the chromatographic steps were not active.

Photon correlation spectroscopy has been used to study aggregation as well as fusion of particles (Goll et al., 1982). A fairly homogeneous liposome population was evidenced at pH 7.4. At pH 5.0 the mean hydrodynamic radius increased as a consequence of the aggregation of the particles. At this pH upon mixing Tb^{+3}- and DPA-liposomes no fluorescence was elicited, demonstrating that aggregation and not fusion of vesicles had occurred. The fusion event was instead triggered by the fusogenic protein, as evidenced by the formation of a large vesicle population. It is known that when spherical vesicles fuse to form a larger spherical vesicle, the fused vesicle has a larger internal volume than the sum of the internal volumes of the constituent vesicles (Nir et al., 1980). However our fusion products appear larger than we would have expected if only dimers had fused. This result should be a consequence of multiple fusion events triggered during the long period of time requested in the laser light scattering measurement.

Generally the extent of leakage during fusion is influenced by the size and composition of liposomes (Bentz & Düzgünes, 1985). Ca^{+2}-induced fusion of large unilamellar phosphatidylserine vesicles is a nonleaky process (Wilschut et al., 1980). Lactalbumin at acidic pH (Kim & Kim, 1986), basic peptides (Bondeson & Sundler, 1990), N-terminal hydrophobic sequence of viral envelope glycoprotein (Martin et al., 1993) produce different extents of leakage during the fusion of liposomes. Fusion triggered by the fusogenic protein purified in our laboratory seems to proceed through a mechanism not accompanied by a detectable leakage of the encapsulated Tb/citrate complex, at least during the first period of fusion (Fig. 3).

When suspensions of liposomes containing phosphatidylcholine are made acidic, the vesicles aggregate, as shown by the increase in light scattering. In this condition aggregation is rapid compared to destabilization of opposed membranes and therefore the fusion step is rate limiting, as deduced by performing the experiments reported in Figs. 4 and 5. Although proteins have been found to be involved in many fusion processes, no kinetics data for protein other than viral structures are available. Fusion rate constant value calculated for our fusogenic protein ($13 \cdot 10^{-3}$ sec^{-1}) is comparable ($4 \cdot 10^{-3}$–$7 \cdot 10^{-3}$ sec^{-1}) with that found for the Ca^{+2}-dependent fusion of liposomes of similar composition (Meers et al., 1988). Fusion rate constant describing kinetics of fusion of influenza virus with liposomes is instead much higher (0.5–1.0 sec^{-1}) (Nir, Stegmann & Wilschut, 1986; Wunderli-Allenspach & Ott, 1990). It is also observed that the rate constant of fusion exhibits the largest variation as a function of pH and liposome com-

position (Nir, Klappe & Hoekstra, 1986). In our experience a decrease of the rate constant was observed when the fusogenic protein was tested at pH higher than 5.0 (*not reported*). The result that *K* is sensitive to pH supports the view that deprotonation of carboxylic residues of the protein influences both hydrophobicity and conformation of the protein and then its affinity for target liposomes. Ca^{+2}-induced fusion of PS/PE or PA/PE liposomes at pH 7.4 is second order, as would be expected for aggregation rate-limiting kinetics. Liposomes of such a composition have been used as a model to study the effect of sinexin on the aggregation step of the overall fusion process (Meers et al., 1988). Since no aggregation was observed upon decreasing the pH from 7.4 to 5.0, we have used PA/PE liposomes to study the first step of the overall fusion. Surprisingly, no second order kinetics has been detected, indicating that the aggregation rate constant is higher than the fusion rate constant-. Therefore fusogenic protein should operate either in the aggregation and fusion steps, aggregation being an event faster than fusion.

A hydrophobicity gradient which permits an oblique orientation in a phospholipid bilayer was found along the axis of the helix of viral fusogenic sequence (Brasseur, 1991). This property seems to be typical of fusogenic proteins (Vanloo et al., 1996). The interaction of our purified fusogenic protein with liposomes (Figs. 8 and 9) suggests the presence of a hydrophobic segment which could penetrate the lipid bilayer. This could explain why the pH did not affect the insertion of the protein into the bilayer, while influencing aggregation and fusion, probably by acting on the protonation of carboxylic groups of the exposed portion of the protein. Other information deduced from the studies on fusogenic protein-liposomes interaction (Fig. 9) concerns the evidence that the glucidic structure is also exposed and probably involved in the modulation of the fusogenic activity (Camici & Corazzi, 1997). The inserted protein, through exposed protonated carboxylic groups, could promote the bridging of the host liposome with an adjacent liposome, followed by aggregation and fusion. This hypothesis is supported by the finding that the protein inserted into unloaded liposomes is not able to promote the fusion of Tb^{+3}- and DPA-liposomes (*data not shown*). On the contrary, if the protein is inserted on DPA-liposomes, it can trigger the fusion with Tb^{+3}-liposomes, as can be argued from the experiment reported in Fig. 8. The proposed mechanism should exclude the possibility of initial fusion to triplets.

In conclusion, we can summarize that a protein associated with the endoplasmic reticulum, possessing both aggregation and fusion properties, has been purified in this work. This finding could be related to the dynamic nature of these membranes. The presence of this protein should reinforce the hypothesis of the membrane contact mechanism for the lipid movement from and to the endoplasmic reticulum. The activity of the fusogenic protein could be triggered by the acidic environment generated by the H^+-ATPase pumps operating in all membranes of the vacuolar system (Rees-Jones & Al-Awqati, 1984). However, the possible ''in vivo'' activity of this protein is in apparent contrast with the known geometry of the intracellular membrane fusion event. Indeed, during the fusion, the cytosol-facing sides of the membranes come in contact with each other in a pH environment near 7.2, being the low pH produced in the internal or noncytosolic compartment of the endoplasmic reticulum. At present time we have no explanation for this topological discrepancy. Our present effort aims to get more information on the structure of the fusogenic protein.

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