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Protein-mediated fusion of liposomes with microsomal membranes of *Aspergillus niger*: evidence for a complex mechanism dealing with membranous and cytosolic fusogenic proteins

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Membrane fusion is a fundamental and wide-spread phenomenon in the functioning of cells. Many studies were carried out concerning fusion of plasma membranes as for example cell-cell fusions or uptake by cells of lipid-enveloped viruses. The present study deals with the interaction of intracellular membranes of *Aspergillus niger* with artificial membranes (liposomes). Association is monitored by the uptake of radioactive liposomes by fungal microsomal membranes. The discrimination between aggregation and pure fusion is done by layering the liposomes-microsomes mixture on a continuous sucrose gradient. The accurate quantitation of the fusion phenomenon is monitored with a fluorescent assay based on resonance energy transfer (Struck, D.K. et al. (1981) Biochemistry 20, 4093–4099). Both methods show that, at physiological pH, there is a spontaneous fusion of microsomes with cholesterol-free liposomes. This phenomenon is protein dependent as trypsinized microsomal membranes are no longer able to fuse with liposomes. Biological significance of the fusion process has been demonstrated using microsomal intrinsic protein mannosylation assay; the enhancement of the lipid to protein ratio due to the fusion of liposomes with microsomes of *A. niger* results in an increase in the rate of endogenous proteins mannosylation. Moreover, cytosolic proteins of *A. niger* promote the fusion of any kind of liposomes with microsomes.

Introduction

Membrane fusion is a puzzling phenomenon which plays an important role in intercellular events like fertilization, myogenesis, hybridization as well as during cell infections by some enveloped viruses and parasites. Intracellular membrane fusion is also essential to explain activities such as endocytosis, membrane traffic and organelle reassembly after mitosis [2]. Numerous studies have been undertaken to find out the factors required for membrane recognition, adhesion and mainly fusion. Non physiological substances have proven to be very useful for the production of hybridomas [3] and monoclonal antibodies [4].

To get a better insight into the phenomenon a more simple model was rapidly chosen with the use of artifical lipid bilayers named liposomes. A

Abbreviations: *N*-Rh-, *N*-(lissamine rhodamine B sulfonyl)-; *N*-NBD-, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-; dol-*P*, dolichyl phosphate; SUV, small unilamellar vesicle.

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great deal of work investigating the molecular requirements for membrane fusion has been undertaken, using liposomes. Special attention has been paid to the fusogenic properties of some divalent cations like Ca^{2+} or Mg^{2+} used at physiological concentrations [5,6] and their influence on various liposomes at low pH [7,8] and in presence of polycations like polyamines [9] or polylysine [10].

Moreover the fusogenic properties of various proteins has been investigated including diphtheria toxin [11], the sperm adhesive protein [12], bovine serum albumin [13] and various viruses fusion proteins [14,15].

The present study deals with the fusion of liposomes of defined lipid composition with intracellular membranes, e.g. microsomes from *Aspergillus niger*, monitored by the measurement of the intermixing of lipids of the two kinds of vesicles. Cholesterol plays a key role in the fusion since cholesterol-containing liposomes fuse far much less than those which are devoid of cholesterol. The phenomenon is modulated by membranous proteins as trypsinized microsomes are unable to fuse any more with the lipid vesicles. The fusion itself can be expressed in biological terms, as we have shown that the fusion of liposomes with microsomes of *A. niger* causes a great enhancement of the well studied intramicrosomal process of proteins mannosylation. Moreover, fungal cytosolic proteins induce the fusion of any kind of lipid vesicles with microsomal membranes of *A. niger*.

Material and Methods

Materials

Aspergillus niger van Tieghem (CBS-126.48) grown on a liquid medium was harvested after 24 h culture and the microsomal and cytosolic fractions were obtained as previously described [16].

Dipalmitoylphosphatidylcholine (DPPC), phosphatidylcholine (egg yolk PC), phosphatidic acid, phosphatidylglycerol, phosphatidylserine (PS) and dolichyl monophosphate (Dol-*P*) were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.). Cholesterol and phosphatidylethanolamine were from Kock light Lab. (Colnbrook, Bucks, U.K.). Radiochemicals were purchased from Amersham International (Amersham, U.K.). *N*-(Lissamine rhodamine B sulfonyl) phosphatidylethanolamine (*N*-Rh-PE) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine (*N*-NBD-PE) were purchased from Avanti Polar Lipids Inc. (Birmingham, AL, U.S.A.).

Trypsin and trypsin inhibitor (egg white) were from Boehringer (Manheim, F.R.G.).

Methods

Preparation of liposomes. Liposomes were freshly prepared before each experiment by sonication, at 40 W under nitrogen, for 15 min at 4°C or at 45°C when dipalmitoylphosphatidylcholine was used. The sonicated suspension was centrifuged 45 min at $100\,000 \times g$ in order to remove the large multilayer liposomes [17]. When necessary, trace amounts of labeled lipids were added to quantify the small unilamellar vesicle (SUV) preparations.

Association assay of liposomes with microsomes. Association was monitored by a method based on the great differences in size and density between liposomes which do not pellet at $100\,000 \times g$ for 45 min and microsomes which, under the same conditions, settle down readily in 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.4). To 100 μl of microsomes (1 mg protein) 50 μl of liposomes (50–75 μg phospholipids) containing 0.4 μCi of cholesteryl [1-^{14}C]oleate (spec. act. 1.85 GBq/mmol) are added in the same buffer. At various times, aliquots of the mixture are centrifuged in an Airfuge (Beckman) for 3 min at $100\,000 \times g$ and the radioactivity in the pellet and the supernatant measured.

Fusion assay of liposomes with microsomes. In the resonance energy transfer (RET) fusion assay, 0.4 mol% of *N*-NBD-PE and 1 mol % of *N*-Rh-PE were incorporated in the lipid bilayer of the liposomes according to Stegman et al. [18]. Measurements were carried out in a final volume of 2 ml 50 mM Tris-HCl buffer (pH 7.4), containing 60 μg of phospholids as lipid vesicles, under continuous stiring. After the addition of 50 μl of microsomes (0.5 mg protein), the increase of *N*-NBD-PE fluorescence due to the dilution of the fluorophores in the microsomal membranes was measured in a Kontron Spectroflurometer SFM.23. The excitation and emission wavelengths were 471

and 520 nm, respectively. For calibration, the initial residual fluorescence of the liposomes was taken as the zero level. The 100% fluorescence was determined by addition of Triton X-100 (5% v/v) to the lipid vesicles suspension or (and) to the liposomes-microsomes mixture with the same subsequent correction of the fluorescence intensity for the sample dilution and for the effect of Triton on the quantum yield of the *N*-NBD-PE [1].

Mannosylation assay of endogenous microsomal proteins. To 100 μl of microsomes (1 mg protein) preincubated with 50 μl of liposomes of various lipid composition (65 μg phospholipids) in 50 mM Tris-HCl buffer (pH 7.4) were added 0.02 μCi of GDP-D-[U-^{14}C]mannose (spec. act. 11 GBq/mmol). The reaction was stopped by the addition of 2 ml of 2:1 (v/v) chloroform/methanol according to Folch et al. [19]. The insoluble material was layered on a glass-fiber filter (Whatman GF/C) and washed by the upper and lower theoretical phases.

Trypsin treatments. 20 mg of microsomal proteins in 20 mM Tris-HCl buffer (pH 7.4) were incubated 15 min at 0°C with 1 mg of trypsin. After incubation, the mixture was centrifuged for 30 min at 100000 × *g*. The pellet, washed and suspended in the same buffer, was referred to as trypsinized microsomes.

4 mg of cytosolic proteins in 20 mM Tris-HCl buffer (pH 7.4) were incubated 15 min at room temperature with 0.2 mg of trypsin. The incubation was stopped by addition of 0.2 mg of egg white trypsin inhibitor.

Other methods. Protein content was estimated according to the biuret procedure [20]. Radioactive samples were assayed with a Packard scintillation Spectrometer Model 300 and counted in 299^{TM} mixture.

Results and Discussion

Artificial membranes like liposomes or natural membranes of *Aspergillus niger* endoplasmic reticulum, taken separately, are stable structures with no tendency to fuse spontaneously; but when mixed together they interact or do not interact depending upon different factors. In a first experiment small unilamellar vesicles (SUV) labeled with radioactive non-exchangeable cholesteryl oleate

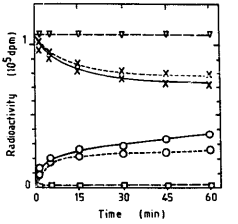

Fig. 1. 'Spontaneous' association kinetics of liposomes with microsomal membranes by the lipid uptake method. 500 μl of lipid vesicles (600–700 μg phospholipids) containing 0.4 μCi of the non exchangeable cholesteryl [1-^{14}C]oleate in 50 mM Tris-HCl buffer (pH 7.4) are mixed with 1 ml of the microsomal suspension (10 mg proteins) in the same buffer. At indicated times, 170-μl aliquots of the mixture are centrifuged 3 min at 100000 × *g* in an Airfuge (Beckman) and the radioactivity of the supernatant solution and the pellet was measured. Liposome composition: (1) phosphatidylcholine/phosphatidylethanolamine/phosphatidic acid (10:5:0.5 mg/ml): × ——— ×, supernatant; ○ ——— ○, pellet; (2) phosphatidylcholine/phosphatidylethanolamine (10:5 mg/ml): × — — — ×, supernatant; ○ — — — ○, pellet; (3) phosphatidylcholine/cholesterol (10:2.5 mg/ml): ▽ — — — ▽, supernatant; □, — — — □, pellet.

[21] were mixed to endoplasmic reticulum vesicles. The difference in size and density between the small unilamellar vesicles (400 Å diameter) and the microsomes (2–4000 Å) was used to separate the free liposomes from the microsomal vesicles associated or not with SUV. This method allows to study the interaction potency of liposomes according to their lipid composition. Fig. 1 shows that neutral or negatively charged liposomes associate spontaneously with microsomes. The phenomenon is fast and reaches a plateau after 30 min. The addition of saturated phospholipids like the dipalmitoylphosphatidylcholine to the lipid composition of the liposomes does not modify their association properties (not shown). The percentage of association of pure phosphatidylcholine or phosphatidylcholine-phosphatidylethanolamine liposomes is the same: 20–25%. The addition of cholesterol decreases the extent of association of such liposomes. This effect is detectable even at a 1 to 10 molar ratio and is not modified by the

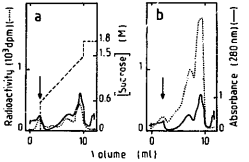

Fig. 2. Discrimination between aggregation or fusion of liposomes with microsomes. Radioactive pellets, obtained after one hour of contact between labeled liposomes and microsomes as in Fig. 1, resuspended in 2 ml 50 mM Tris-HCl buffer (pH 7.4) are layered on 8 ml of a continuous 0.6–1.5 M sucrose gradient over a cushion of 1.8 M sucrose (2.5 ml). Control data are obtained by layering 2 ml of liposomes. After 14 h centrifugation at 170000 × *g* in a SW 41 rotor (Beckman), the gradient was collected using a density gradient fractionator 640 (Isco). The absorbance is monitored at 280 nm (Isco UA-5 Unit) and the radioactivity measured as described in Methods. (a) Microsomes + cholesterol-containing liposomes (phosphatidylcholine/cholesterol (10 : 2.5 mg/ml)). (b) Microsomes + cholesterol-free liposomes (phosphatidylcholine/phosphatidylethanolamine (10:5 mg/ml)). ———, absorbance; ·····, radioactivity; ←, liposomes alone.

addition of negatively charged phospholipids like phosphatidylserine or phosphatidic acid (results non shown). The extent of association is somewhat higher with negatively charged lipid vesicles with a slight advantage to phosphatidylglycerol-containing liposomes.

In order to discriminate between a possible aggregation and fusion, several experiments were carried out. Liposomes alone or the liposomes and microsomes mixture were layered over a continuous sucrose gradient and centrifuged in a SW 41 rotor (Beckman) for 14 h at 170000 × *g*. The results of this experiment which is a modification of the method used by Haywood and Boyer [22] are shown in Fig. 2. Free liposomes stay at the top of the gradient whereas associated lipid vesicles co-migrate with the microsomal fraction. The same difference of the extent of association is observed depending upon the presence or the absence of cholesterol in the lipid composition of liposomes. Only 3% of the starting radioactive liposomes containing cholesterol remained associated with the microsomal fraction whereas 17% are found associated with the microsomes when the starting liposomes are cholesterol-free.

The resonance energy transfer (RET), method which gives an idea of the intermixing of lipids during the membranes fusion, was used to check this phenomenon. Upon fusion of the fluorescent liposomes with microsomal membranes the efficiency of energy transfer is reduced since lateral diffusion of the energy acceptor in the plane of newly formed membranes lowers its surface density [1]. The results of Fig. 3 show the high percentage of fusion of cholesterol-free liposomes

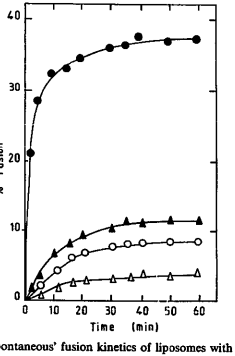

Fig. 3. 'Spontaneous' fusion kinetics of liposomes with microsomal membranes monitored by resonance energy transfer (RET). To 50 μl of liposomes (80 μg phospholipids) containing the fluorescence probe *N*-NBD-PE and *N*-Rh-PE in 2 ml of 20 mM Tris-HCl buffer (pH 7.4) are added 50 μl of the microsomal suspension (0.8 mg proteins) in the same buffer. The increase of the *N*-NBD-PE fluorescence due to the dilution of the fluorophores in the microsomal membranes is monitored at 520 nm. For calibration, the initial residual fluorescence of the liposome is taken as the zero level. The 100% fluorescence is determined by addition of Triton X-100 (5% v/v) [18]. Liposomes: phosphatidylcholine/phosphatidylethanolamine/phosphatidic acid/*N*-NBD-PE/*N*-Rh-PE (10:5:1:0.04:0.016 mg/ml): ●——●, liposomes + microsomal membranes; ▲——▲, liposomes + trypsinized microsomal membranes. Liposomes: phosphatidylcholine/dipalmitoylphosphatidylcholine/cholesterol/*N*-NBD-PE/*N*-Rh-PE (6:6:4:0.08:0.016 mg/ml): ○——○, liposomes + microsomal membranes; △——△, liposomes + trypsinized microsomal membranes.

with microsomal membranes. The membrane intermixing, in the case of cholesterol-containing vesicles, is very low. Liposomes of various lipid compositions were tested with the RET technique. The results observed are similar to those obtained with the labeled lipid uptake method. The ability of SUV containing the fluorescent probes to fuse with liposomes devoid of such probes was tested; in all cases we never observed any fluorescence variation. The biological membranes are not only composed of lipids they also contain proteins which can play a major role in membranes fusion as they can be implicated either in recognition or (and) adhesion processes. Indeed, in the experiment shown in Fig. 3, we observed that the trypsination of the microsomes (cf. Methods) causes the abolition of fusion with liposomes irrespective of their lipid composition. The same observation was made using he labeled lipid uptake technique as in Fig. 1. We have checked that the trypsin and the trypsin inhibitor had no effect on the fusogenic potency of liposomes of any kind.

All the methods used gave the same result: liposomes devoid of cholesterol fuse 'spontaneously' with microsomal vesicles. The level of the 'spontaneous' fusion depends of the ratio SUV/microsomal vesicles and the fusion phenomenon itself seems to be finite. In other words, the quantity of phospholipid vesicles that can mix with the bilayers of the microsomes is limited. This observation differs marquedly from the usual liposomal model where the fusion process will continue until all lipid vesicles are transformed into giant structures [23]. The complexity of the phenomenon is emphasized by the existence of microsomal membranes proteins which mediate the fusion of microsomes with liposomes and which can discriminate between the type of lipid vesicles allowing or not fusion. This discrimination is based on the presence of cholesterol which plays a key role either by a direct interaction with the microsomal proteins involved or by its effect on membrane packing, viscosity [24,25] and membrane stability due to its special ordering effect on the membrane lipids [26] in the neighbourhood of the implicated proteins of the reticulum. These proteins could also recognize some physical state differences due to the presence or absence of cholesterol in the liposomes composition allowing the contact but not the fusion itself.

Does the fusion of liposomes with microsomes have a biological effect or significance? to investigate this point we have studied the effect of the fusion of microsomes with negatively charged liposomes of various lipid compositions on the mannosylation of endogenous proteins of the endoplasmic reticulum. This well understood in-

TABLE I

0.9 ml of microsomal membranes suspension (18 mg protein) are mixed together with 0.4 ml of liposomes (5 mg phospholipids) in 25 mM Tris-HCl buffer (pH 7.8), 4 mM $MgCl_2$. At zero time 0.5 μCi of GDP-D-[U-^{14}C]mannose is added to the mixture. After 5 or 45 min of incubation the reaction medium is centrifuged for 30 min at 100000 × g. The pellet and the supernatant are extracted by the chloroform-methanol (2:1, v/v) mixture according to Folch et al. [19]. The organic lower phase thus obtained is washed by the theoretical upper phase and counted. The interphase containing the denatured proteins is filtered, washed and its radioactivity measured.

Liposomes composition (mg/ml)	Time (min)	Supernatant Radioactivity (dpm) associated to the lipids	Pellet Radioactivity (dpm) associated	
			to the lipids	to the proteins
Phosphatidylcholine/ phosphatidylglycerol/ phosphatidic acid (10:3:0.2)	5	450	82500	180000
	45	2100	128000	275000
Phosphatidylcholine/ phosphatidylglycerol/ dolichyl phosphate (10:3:0.4)	5	3800	454000	275000
	45	16000	350000	332000

tramembranous process in *Aspergillus niger* [16,27], proceeds as follows:

GDP-mannose + Dol-*P* ⇌ GDP + Dol-*P*-mannose
Dol-*P*-mannose + protein → protein-mannose + Dol-*P*

Table I confirms the membranous character of the reaction since the radioactivity of the lipids of the supernatant never exceeds 2.5% of that found in the microsomes. Therefore no mannose was transfered to the dolichyl phosphate of the free liposomes found in the supernatant. The synthesis of dolichyl phosphate-mannose occurs only after the mixing of the lipids of the liposomes with those of the microsomes i.e. after their prerequisite

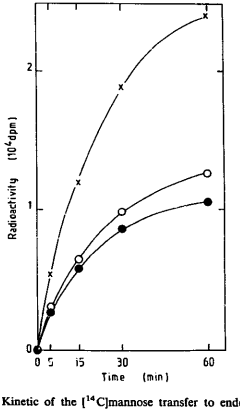

Fig. 4. Kinetic of the [^{14}C]mannose transfer to endogenous microsomal proteins after liposome fusion. 0.6 ml of microsomes (10 mg proteins) in 50 mM Tris-HCl buffer (pH 7.4) are incubated with 0.5 ml of liposomes (6–8 mg phospholipids), or buffer (control) for 30 min at room temperature as in Fig. 1. Then 0.1 μCi of GDP-D-[U-^{14}C]mannose is added to the mixture. At various time-intervals, aliquots of the suspension are withdrawn and the radioactivity of the washed proteins pellet measured (cf. Methods). ●——●, microsomes + ;liposomes (phosphatidylcholine/cholesterol (10 : 2.5 mg/ml)); ×——×, microsomes + liposomes (phosphatidylcholine/-phosphatidylethanolamine/phosphatidic acid (10 : 5 : 1 mg/ml)). ○——○, control.

fusion. Again we observe (Fig. 4) the same difference depending upon the presence or absence of cholesterol: when cholesterol or ergosterol (not shown)-containing liposomes are used, the proteins mannosylation is at the same level as that in the assay carried out in the absence of lipid vesicles. Lipid vesicles, free of cholesterol, cause a dramatic enhancement of the initial velocity of the mannosylation of endogenous proteins up to 3-fold higher than normally but have no effect on the final level since the mannosylation sites are limited (Fig. 4 and Table I). This last result differs markedly from the strong inhibitory effect of phosphatidylcholine and phosphatidylethanolamine on membrane-bound brain fucosyltransferase [28]. Anyhow, the modulation of the mannosyltransferase seems to be a direct consequence of the lipid enrichment of the microsomal vesicles and not a modification of the enzyme affinity since the fusion event does not change the K_m value but enhances the V_m (not shown).

Some preliminary experiments have lead us to consider the effect of the cytosol on the fusion phenomenon. The fusion assay was modified in order to avoid any 'spontaneous' fusion. Therefore the incubation medium was composed of liposomes choosen for their high reproducibility and very low fusogenic potency of the following composition: phosphatidylcholine (egg yolk)/dipalmitoylphosphatidylcholine/cholesterol (6 : 6 : 4), and trypsinized microsomes (cf. Methods). The mixing of lipids monitored by the RET technique which under these conditions is very low, was greatly enhanced by the addition of the cytosol of *Aspergillus niger*. To get a better insight of the nature of the soluble fusogenic substance, the cytosol was chromatographed on DEAE-cellulose. Three main fractions were collected. Only the fraction eluted with 130 mM NaCl (pH 7.4) (20% of total proteins) proved to be active. Fig. 5 shows the evident fusogenic properties of such a fraction on a mixture consisting of trypsinized microsomes and cholesterol-containing liposomes. The fusogenic properties of the cytosol and of the fraction eluted from the DEAE-cellulose column with 130 mM NaCl are abolished by heating and trypsination, indicating the protein nature of the fusogens present in the cytosol of *Aspergillus niger*.

In the last part of this report we have demon-

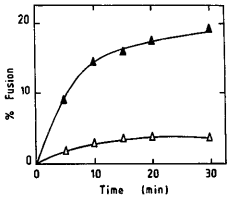

Fig. 5. Fusion of cholesterol-containing liposomes with trypsin-treated microsomes in the presence of a cytosolic fraction from *Aspergillus niger*. To 50 μl of liposomes (80 μg phospholipids) of the following composition: phosphatidylcholine/dipalmitoylphosphatidylcholine/cholesterol/*N*-NBD-PE/*N*-Rh-PE (6:6:4:0.08:0.016 mg/ml), in 2 ml Tris-HCl buffer (pH 7.4) are added 50 μl of trypsinized microsomes (1 mg proteins). At zero time, 0.1 mg of the cytosolic fraction eluted from a DEAE-cellulose column by 130 mM NaCl in 20 mM Tris-HCl buffer (pH 7.4) or the same volume of buffer (control) are added to the mixture liposomes + microsomes. Both samples are excited at 470 nm and the emission fluorescence is monitored at 520 nm. The kinetics are obtained as for Fig. 3. △———△, control; ▲———▲, + cytosolic fraction eluted from DEAE-cellulose column.

strated the existence of acidic cytosolic proteins able to induce fusion of lipid vesicles of various lipid compositions with microsomes pretreated with trypsin. So far, few works dealing with the fusogenic properties of soluble cationic proteins like lysin [29], vesicle-bound lysozyme at acidic pH [30] or mellitin [31] with sonicated lipid vesicles have been reported. Recent studies have also shown the Ca^{2+} or pH dependent fusion capability of synexin [32] or clathrin [33] on liposomes. In this last case, the crucial role of the protein hydrophobicity in the initiation of membrane fusion has been suggested [23]. To summarize, the fusogenic potency of the mentionned proteins is either due to a general requirement for the proteins to be positively charged when negatively charged phospholipid vesicles are used, or to their amphiphatic nature [34]. Moreover an interesting approach is the demonstration [35] of the per se fusogenic properties of soluble lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase on acidic lipid vesicles.

Do cytosolic protein(s) have any similarity with hydrophobic or Ca^{2+}-dependent phospholipid-binding proteins that mediate tight intermembranes contact and thus can regulate fusion [36,37]? This point is important because of the existence within the cytosol of *Aspergillus niger* of such phosphatidylcholine-binding protein [38]. Further work is needed to isolate and characterize such proteins.

Conclusion

In this study, we have pointed out that the fusion phenomena involve two kinds of proteins: membraneous fusogenic proteins from the endoplasmic reticulum which seem able to recognize some type of lipid or at least some physical state of the lipid vesicles and thus allowing or not the fusion. This special fusogenic behaviour of membranes of the rough endoplasmic reticulum has already been observed by J. Paiement [39] who concluded that such membranes, nuclear envelope and Golgi membranes possess unique recognition and GTP or ATP/divalent cation-mediated fusion properties which are due to cytosolically exposed proteins [40]. This last conclusion has been recently confirmed by the transfer of liposome-encapsulated macromolecules into isolated mouse liver nuclei, at acidic pH [30].

The second type are protein(s) from the cytosol of *Aspergillus niger* that trigger the fusion of any type of lipid vesicles with microsomes. Further studies will tell us about the structure and function of soluble fusogenic proteins as one can consider the reassembly of Golgi, rough endoplasmic reticulum or nuclear membrane vesicles after mitosis [2]. However, the knowledge of cell compartmentation, organelle division and membrane traffic and recycling must be reappraised according to the cellular fusion events mediated by membranous and (or) soluble fusogenic proteins.

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