

# Lens Major Intrinsic Protein (MIP) Promotes Adhesion When Reconstituted into Large Unilamellar Liposomes<sup>†</sup>

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**ABSTRACT:** The vertebrate lens behaves like a syncytium, and it is formed mainly by cells called lens fibers. Between the fibers are extensive networks of membrane junctions. The major intrinsic protein (MIP) constitutes about 50–60% of the intrinsic membrane proteins found in lens fiber junctions. The role of MIP is unknown. Nevertheless, it has been proposed that it is the protein responsible for the adhesion between the plasmatic membranes of the lens fibers. The aim of our studies was to test the adhesion-promoting role of MIP. We reconstituted MIP into large unilamellar vesicles (LUV) of phosphatidylcholine (PC) and studied the vesicle aggregation between MIP-reconstituted LUV (PC-MIP) and phosphatidylserine (PS) vesicles. The aggregation process was monitored using methods based on resonance energy transfer (RET) and turbidity measurements. Neither RET nor an increase in turbidity occurred in any combination except in the presence of both MIP and PS. The liposomes thus aggregate through protein–lipid interactions. These results show that MIP promotes adhesion with negatively charged membranes, indicating that the adhesion is electrostatic in nature. Aggregation was fastest at pH 6.0. The aggregation effect was abolished with pronase treatment. Preincubation of PC-MIP vesicles with anti-MIP polyclonal serum also inhibited the aggregation. These studies are the first experimental evidence supporting the hypothesis of an adhesive role for MIP.

The vertebrate lens is composed of a mass of extremely elongated, closely apposed cells called fibers, which are both metabolically and electrically coupled (Mathias & Rae, 1985). These hexagonal cells make contact with each other through numerous membrane appositions known as fiber junctions, which resemble gap junctions in displaying pentalamellar structure. Morphological studies reveal two kinds of fiber junctions: one about 11–13 nm thick (found more frequently toward the nucleus of the lens) and the other about 18–20 nm wide (more abundant between cortical cells). Both types of junction differ in protein composition and structure. Immunocytochemical studies have shown that the main intrinsic protein (MIP,<sup>1</sup> 28 kDa), which constitutes about 50–60% of the plasma membrane protein (Benedetti et al., 1976; Bloemendal et al., 1977), is present in 11–13-nm junctions. The pentalamellar structure of both junctions originally led to the postulation that they were analogous to the gap junctions described in other organs and served a similar function in communicating between the lens fibers. However, MIP is not structurally related to the connexins (Bennett et al., 1991). Freeze-fracture studies indicate that MIP molecules are present in only one of the membranes of the junction, forming a tetragonal particle array; from there, the protein abuts a particle-free membrane (Lo & Harding, 1984; Zampighi et

al., 1989). This is in sharp contrast to the structure of gap junctions, where two hemichannels (connexons) span both cell membranes. Finally, the intercellular space is much narrower than that in gap junctions (0.5–0.7 vs 3–5 nm). Thus, the 11–13-nm junctions are not gap junctions, and MIP is probably not directly communicating between the cytoplasm of lens fibers.

It has been proposed instead that MIP promotes cell-to-cell adhesion and collapses the intercellular space due to its channel-forming properties (Zampighi et al., 1989). This would allow the formation of the 11–13-nm junctions with their extremely small intervening spaces. Since hydrophobicity plots predict that MIP leaves three small extracellular loops with net positive charge, the possible adhesive role of MIP can be explained by assuming that this positive domain interacts with negatively charged lipids of the opposing membrane (Zampighi et al., 1989, 1992).

The purpose of the present study was to test this hypothesis. We assayed both total junction proteins and pure MIP reconstituted in neutral PC liposomes and studied the interaction of these liposomes with negatively charged liposomes prepared from PS. The association of the vesicles was determined by resonance energy transfer (RET) and turbidity assays. We found that PC liposomes containing MIP bound PS liposomes and that this process depended absolutely and specifically upon the presence and integrity of the MIP. These studies thus lend support to the hypothesis of an adhesive role for MIP in mammalian lens.

## EXPERIMENTAL PROCEDURES

**Materials.** Phosphatidylserine (L- $\alpha$ -phosphatidylserine brain sodium salt) (PS) and phosphatidylcholine (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) (PC) were purchased from Avanti Polar Lipids (Birmingham, AL). Rhodamine-phosphatidylethanolamine (Rh-PE) and NBD-phosphatidylethanolamine (*N*-(7-nitro-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) (NBD-

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<sup>1</sup> Abbreviations: MIP, major intrinsic protein; LUV, large unilamellar vesicles; Mbs, lens junctional membranes; RET, resonance energy transfer; PS, phosphatidylserine (L- $\alpha$ -phosphatidylserine brain sodium salt); PC, phosphatidylcholine or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Rh-PE, rhodamine-phosphatidylethanolamine; NBD-PE, NBD-phosphatidylethanolamine or *N*-(7-nitro-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-morpholinopropanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

PE) were purchased from Molecular Probes (Eugene, OR). Pronase from *Streptomyces griseus* was obtained from Calbiochem (La Jolla, CA). Octyl  $\beta$ -glucopyranoside was purchased from Sigma Chemical Co. (St. Louis, MO). Low molecular weight protein standards were purchased from Bio-Rad. All inorganic salts, reagents, or buffers were analytical reagent grade or better.

**Isolation of Lens Fiber Junctions.** Lenses from adult bovines were obtained at the slaughterhouse, immediately cooled on ice, and stored at  $-20^{\circ}\text{C}$  until use. The junctions were isolated using a previously described procedure (Ehring et al., 1992). Briefly, the capsules of eight lenses were removed, and the lenses were cut into small pieces with a razor blade and homogenized in 15 mL of solution A (2 mM  $\text{NaHCO}_3$ , 3 mM EDTA, and 100  $\mu\text{M}$  PMSF, pH 8.0). The suspension was filtered through eight layers of surgical gauze, diluted to 60 mL with solution A, and centrifuged at 3640g for 15 min. The pellet was washed and centrifuged twice in the same solution and then resuspended in solution B (5 mM EDTA, 1 mM  $\text{CaCl}_2$ , 1.5 mM  $\text{NaN}_3$ , and 4 mM Tris, pH 8.0) in 4 M urea. The suspension was centrifuged at 22500g for 25 min. The white portion of the pellet was extracted with 50 mL of solution B in 7 M urea and centrifuged at 39000g for 45 min. The pellet was washed three times with 50 mL of solution B and centrifuged at 100000g for 10 min. After each centrifugation, the dark central region of the pellet was discarded. The isolated junctions were diluted in solution B plus 10% glycerol and stored at  $-20^{\circ}\text{C}$ .

**Solubilization of Lens Fiber Junction Proteins.** Plasma membranes were solubilized by adding octyl  $\beta$ -glucopyranoside to the lens fiber junction suspension (25 mg of detergent per mg of junctional protein) and sonicated for 30 s. The clear solution was centrifuged at 14000g for 5 min, and the supernatant was filtered through a 0.2- $\mu\text{m}$  PVDF filter (Acro Disc, Gelman Sciences, Ann Arbor, MI).

**Purification of MIP.** MIP was purified from the lens fiber junction membranes using the method described by Ehring et al. (1992). Briefly, 500  $\mu\text{L}$  of solubilized membranes (300–400  $\mu\text{g}$ ) was injected into an analytical MA7Q column (Bio-Rad) and eluted with a linear gradient (0.025–0.5 M NaCl in buffer A in the presence of 34 mM octyl  $\beta$ -glucopyranoside). Protein was detected by its absorbance at 280 nm. MIP eluted with a retention time of 39 s. This fraction exhibited a single band with an apparent molecular weight of 28 000 in 12.5% SDS-PAGE. The protein was stored frozen after being concentrated to 0.5–0.7 mg/mL using microconcentrator tubes (Centricon 10, Amicon, Danvers, MA).

**Preparation of Large Unilamellar Vesicles of Phosphatidylserine.** Large unilamellar vesicles (LUV) were prepared by the extrusion procedure (Hope et al., 1985; MacDonald et al., 1991). Phosphatidylserine (PS) in chloroform was dried under a nitrogen stream and desiccated thoroughly under vacuum. The dried lipids were hydrated with 200  $\mu\text{L}$  of reconstitution buffer (100 mM NaCl and 25 mM MOPS, pH 7.0) for 30 min at room temperature. The lipid was suspended by vortexing for 1 min and was then immediately extruded through a 0.1- $\mu\text{m}$  polycarbonate filter (Costar Nuclepore, Pleasanton, CA). The lipid concentration was determined in all preparations by a phosphate assay (Meek, 1986). PS LUV containing either Rh-PE or NBD-PE were prepared in the same way, adding the fluorescent probe to the initial solution of PS at a concentration of 2 mol %. The resulting vesicles are called Rh-PS or NBD-PS.

**Protein Reconstitution into Large Unilamellar Vesicles of Phosphatidylcholine.** The solubilized junctional proteins or

purified MIPs were reconstituted into phosphatidylcholine LUV using the dialysis method described by Mimms et al. (1981), and the resulting liposomes were sized by extrusion through 0.1- $\mu\text{m}$  Nuclepore filters. Vesicles were reconstituted at different lipid/protein ratios (expressed in micrograms): 1000/500, 1000/400, 1000/375, 1000/250, 1000/200, 1000/100, 1000/50, and 1000/25. We observed adhesive properties using ratios above 1000/250. All of the experiments described in this article were performed using a ratio of 1000/500. Phosphatidylcholine (1 mg) in chloroform was dried under a nitrogen stream and suspended in 0.1 mL of reconstitution buffer plus octyl  $\beta$ -glucopyranoside (112 mg/mL). When fluorescent vesicles were prepared, 2 mol % of either NBD-PE or Rh-PE was added. The solution was mixed with solubilized junctional proteins (500  $\mu\text{g}$ ) or purified MIP (400  $\mu\text{g}$ ) and sonicated for 30 s. This clear solution was dialyzed against 3 L of reconstitution buffer (three changes, 26 h at  $4^{\circ}\text{C}$ ), and the resultant whitish suspension was filtered through a 0.1- $\mu\text{m}$  polycarbonate filter. Protein (Bradford 1976) and phospholipid (Meek, 1986) contents were measured, and the protein composition of the reconstituted vesicles was analyzed by SDS-PAGE (12.5% or 6–22% gradient gels). Under our experimental conditions,  $56 \pm 16\%$  ( $\pm\text{SD}$ , eight different preparations) of the junctional membrane protein was incorporated into liposomes.

The presence of the junctional proteins on PC vesicles was studied using SDS-PAGE. As in lens junction membranes, the most abundant protein reconstituted was MIP, being about 60% of the total protein (data not shown).

The vesicles are called PC-Mbs when reconstituted with total junction proteins or PC-MIP when reconstituted with purified MIP. These names are preceded by NBD or Rh when fluorescent probes are also present in the LUV (i.e., NBD-PC-Mbs).

**Resonance Energy Transfer Assay.** This method is based on the fact that the fluorescent emission of NBD-PE in a population of liposomes can be quenched due to resonance energy transfer (RET) to Rh-PE in another population of liposomes if the vesicles come close together (Duzgunes et al., 1987). Experiments were carried out at  $25^{\circ}\text{C}$  in cuvettes containing 3 mL of 100 mM NaCl and 25 mM Bis-Tris (pH 6.0). Emission of NBD-PE-containing liposomes (usually 25  $\mu\text{M}$  PS or PC) was followed at 520 nm (excitation at 450 nm) for 2 min, and then the Rh-PE-labeled liposomes were added (25  $\mu\text{M}$  PC or PS).

**Turbidity Assay.** These experiments were carried out in 1-mL cuvettes under the same conditions used in the RET experiments. Absorbance at 350 nm was measured in a Gilford 2400 spectrophotometer (Gilford Systems, Oberlin, OH) and recorded on a Perkin-Elmer 56 recorder (Perkin-Elmer Ltd., Tokyo, Japan).

**Preparation of anti-MIP Antibody.** Polyclonal anti-MIP antibody was prepared in rabbits by immunization with 500  $\mu\text{g}$  of HPLC-purified MIP according to Harlow and Lane (1988). Western blot analysis of junctional membrane proteins and pure MIP isolated by SDS-PAGE was carried out according to Towbin et al. (1979) and developed by the enhanced chemiluminescence method (Amersham, Little Chalfont, Buckinghamshire, UK).

## RESULTS

**Evidence for the Aggregation of PC-Mbs with PS Large Unilamellar Vesicles (LUV).** (i) **Fluorescence Assays.** Electronic excitation energy can be efficiently transferred between a fluorescent energy donor and a suitable energy acceptor.

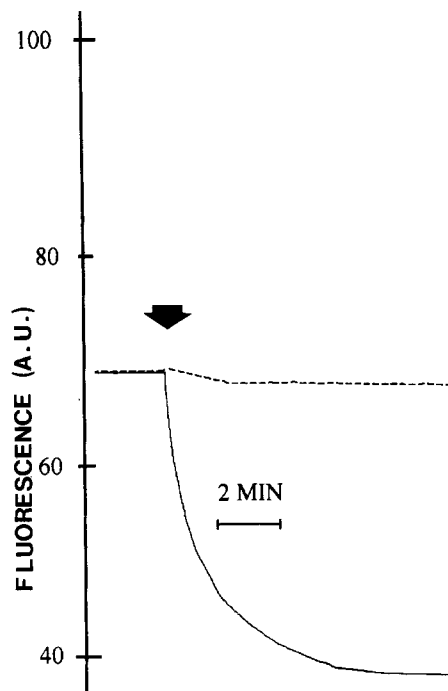


FIGURE 1: Aggregation of PC-Mbs with PS vesicles detected by fluorescence assay. NBD-PC-Mbs or NBD-PC vesicles were preincubated in 100 mM NaCl and 25 mM Bis-Tris (pH 6) at 25 °C. After 3 min, Rh-PS vesicles were added to the cuvette (arrow). The time course of NBD fluorescence emission was recorded (excitation, 450 nm; emission, 520 nm; slit width, 10 nm): mixture of NBD-PC-Mbs and Rh-PS vesicles (—); mixture of NBD-PC and Rh-PS vesicles (- - -).

This process is known as resonance energy transfer (RET) and depends on the distance between the two fluorescent probes (Stryer, 1978). Different RET pairs (donor and acceptor) have been used; in our experiments, we used NBD (donor) and Rh (acceptor).

In order to investigate the role of MIP as a membrane adhesive promoting agent, NBD-PC-Mbs and Rh-PS vesicles were assayed using the probe mixing method. Here, the NBD fluorescence decreases as consequence of RET to Rh (Duzgunes et al., 1987).

Figure 1 shows a typical emission time course at 520 nm of NBD-PC vesicles reconstituted with lens junction solubilized proteins. Immediately after the addition of Rh-PS vesicles (arrow), a rapid fall in the NBD emission occurs, reaching a plateau within 10 min. The amount of quenching was 57% (38, arbitrary units) of the initial fluorescence (69.3, arbitrary units). There is no quenching effect after the mixing of NBD-PC and Rh-PS vesicles, nor after mixing NBD-PC-Mbs with Rh-PC, NBD-PC with Rh-PC-Mbs, or NBD-PC-Mbs with Rh-PC-Mbs vesicles (data not shown). The quenching of NBD emission was seen only when LUV reconstituted with junctional proteins were mixed with PS vesicles.

The presence of the junctional proteins on PC-reconstituted vesicles was studied using SDS-PAGE. As in lens junction membranes, the most abundant protein reconstituted was MIP, being about 60% of the total protein (data not shown).

To prove that our assay was measuring vesicle aggregation, not fusion or lipid mixing, we showed that neither of these occurred between the vesicles. In this experiment (Duzgunes et al., 1987), both probes, NBD-PE and Rh-PE, were incorporated into PS vesicles and then mixed with unlabeled PC-Mbs vesicles. If fusion and/or lipid mixing were to occur, the donor emission would rise as a result of decreasing RET

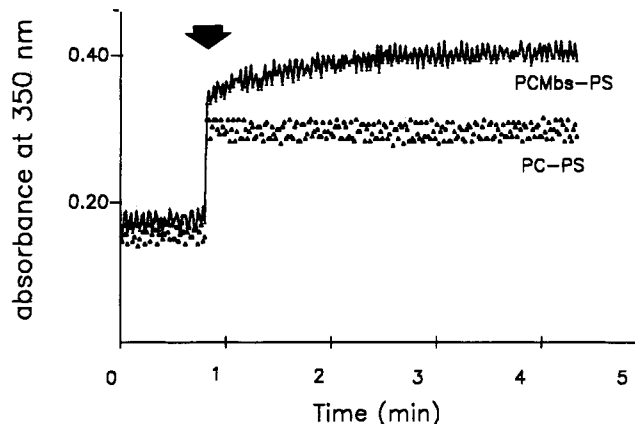


FIGURE 2: Aggregation of PC-Mbs with PS vesicles detected by the turbidity assay. The absorbance time course at 350 nm was monitored when PC-Mbs vesicles (—) and PC vesicles (▲) were mixed with 25  $\mu$ M PS vesicles. Vesicles were incubated in the same solution described for Figure 1. After 3 min of preincubation, 25  $\mu$ M PS vesicles was added to the cuvette (arrow).

efficiency. In five experiments using the probe dilution assay, no change in the NBD emission was observed, although the turbidity assay (see below) revealed that aggregates were forming. Thus, the interaction between junctional proteins and PS membranes is adhesive only and does not destabilize membranes to cause membrane mixing or fusion.

(ii) *Turbidity Assays.* Because the apparent absorbance depends on the LUV size and concentration, the formation of dimers or higher aggregates can be monitored following the increase in absorbance at 350 nm. Figure 2 shows a typical time course of aggregation between PC-Mbs and PS LUV. After the addition of PS vesicles, there is an instantaneous increment in the absorbance, due to the increase in particle concentration (25 to 50  $\mu$ M). Thereafter, a slow increase in absorbance reveals the formation of aggregates. As in RET experiments, the aggregation was dependent on the presence of junctional proteins, since mixing of PC and PS liposomes did not give aggregation, as shown by the unchanging absorbance after the initial rise (Figure 2). Also, in agreement with RET probe mixing experiments, no formation of aggregates was observed between PC-Mbs and PC vesicles or between PC-Mbs and PC-Mbs liposomes.

(iii) *Effect of PC-Mbs LUV on Ca<sup>2+</sup>-Induced PS Vesicle Aggregation.* It has been shown previously that Ca<sup>2+</sup> in the millimolar range induces massive aggregation and fusion of PS vesicles (Lansman & Haynes, 1975; Duzgunes & Ohki, 1977; Portis et al., 1979). In order to gain some insight into the physical interactions between phosphatidylserine and the junctional membrane proteins in the reconstituted LUV, we measured the aggregation induced by 5 mM Ca<sup>2+</sup>. Figure 3 shows that after 5 min of preincubation of the PC and PS vesicles, following Ca<sup>2+</sup> addition (black arrow), a rapid increase in the absorbance was observed. The presence of junctional membrane proteins in the PC vesicles shows an inhibitory effect on Ca<sup>2+</sup>-induced aggregation. This effect was dependent on the amount of PC-Mbs vesicles added to the assay. A 7-fold excess of PC-Mbs inhibits the Ca<sup>2+</sup>-induced massive aggregation completely.

(iv) *pH Aggregation Dependence.* The initial rate of quenching was obtained by measuring the slope of the first 30 s after the mixing of LUV populations (see Figure 1). This parameter was used to quantify the initial rate of aggregation under various experimental conditions. The effect of pH on the initial rate of quenching at various pH conditions was studied. Figure 4 shows the profile in a pH range of 5.0–8.0.

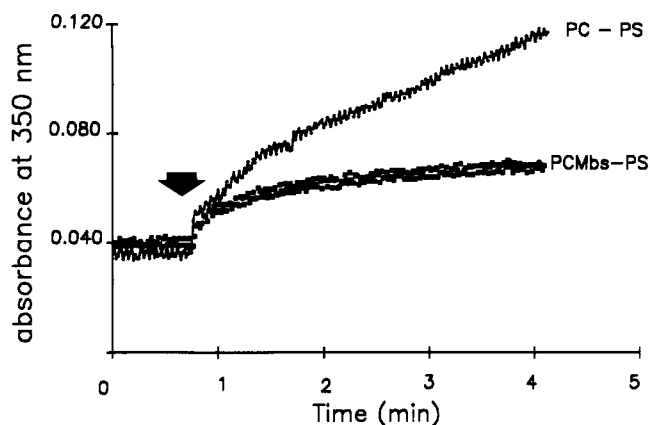


FIGURE 3: Effect of PC-Mbs vesicles on  $\text{Ca}^{2+}$ -induced PS vesicle aggregation. PS vesicles were mixed with PC-Mbs vesicles (■). After 10 min of preincubation, 5 mM  $\text{Ca}^{2+}$  was added to the cuvette (arrow). Aggregation was monitored at 350 nm. The solid line shows the time course of aggregation between PS vesicles mixed with PC vesicles after the addition of 5 mM  $\text{Ca}^{2+}$ .

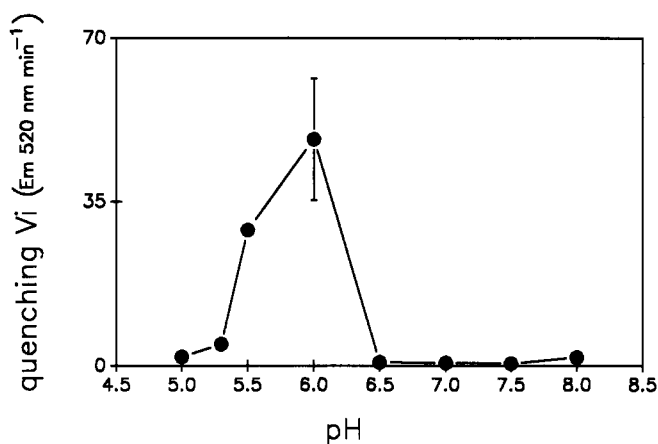


FIGURE 4: Effect of pH on aggregation between PC-Mbs and PS vesicles. NBD-PC-Mbs vesicles were mixed with Rh-PS vesicles in 100 mM NaCl at 25 °C, in the presence of buffers at different pH. Buffers used: 25 mM Bis-Tris (pH 5.0–6.5); 25 mM MOPS (pH 7.0), and 25 mM HEPES (pH 7.5 and 8.0). The initial rate of quenching ( $V_i$ ) was obtained as described in Results. Each point represents the mean  $\pm$ SD of 3–8 independent determinations from different preparations.

The initial rate of quenching increases steeply from pH 5.3, reaching a maximum at pH 6.0. A sharp decline was observed at pH 6.5. The same profile was obtained when pure MIP was used in the LUV reconstitution.

(v) *Effect of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on LUV Aggregation.* The aggregation effect was observed only when LUV reconstituted in the presence of junctional membrane proteins were mixed with PS vesicles. Phosphatidylserine is a negatively charged phospholipid. This suggests that an electrostatic interaction between MIP and PS is involved in the LUV aggregation. Binding of cations to acidic phospholipid vesicles results in charge neutralization and vesicle aggregation (Nir et al., 1983). Regarding these observations, it was of interest to test the effect of divalent cations on the initial rate of quenching. Figure 5A shows the time course of NBD-PC-Mbs emission of vesicles preincubated at 0 mM  $\text{Ca}^{2+}$  and in presence of 5 mM  $\text{Ca}^{2+}$ . The presence of  $\text{Ca}^{2+}$  produces a significant inhibition of quenching. In a similar manner, preincubation of NBD-PC-Mbs vesicles with  $\text{MgCl}_2$  at different concentrations also reduces the initial rate of quenching (Figure 5B).

*MIP-Induced LUV Aggregation. (i) Effect of anti-MIP Polyclonal Antibody on PC-Mbs-Induced Aggregation.* In

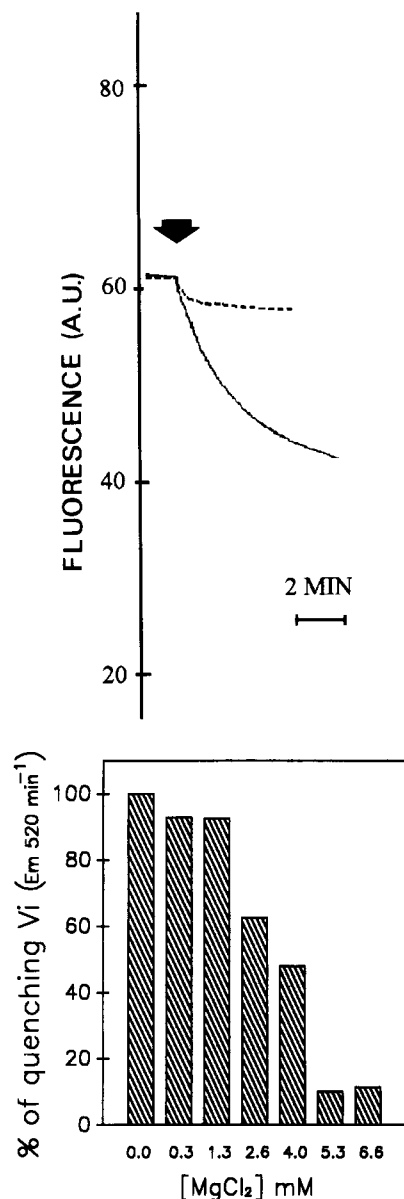


FIGURE 5: Effect of divalent cations on aggregation between PC-Mbs and PS vesicles. (A, top) NBD-PC-Mbs (25  $\mu\text{M}$ ) vesicles were preincubated at 0 mM  $\text{Ca}^{2+}$  (—) and in the presence of 5 mM  $\text{Ca}^{2+}$  (---) for 2 min (100 mM NaCl and 25 mM Bis-Tris (pH 6.0) at 25 °C) and then mixed (arrow) with Rh-PS vesicles (25  $\mu\text{M}$ ). The time course of NBD fluorescence emission was recorded as for Figure 1. (B, bottom) As described in A, NBD-PC-Mbs vesicles were preincubated with  $\text{MgCl}_2$  at concentrations ranging from 0 to 6.6 mM. Each column represents the percentage of the initial rate of quenching obtained from the slope of the NBD emission time course at 0 mM  $\text{Mg}^{2+}$ .

order to establish that MIP is the intrinsic protein responsible for the LUV aggregation, we incubated NBD-PC-Mbs vesicles with a polyclonal antibody against HPLC-purified MIP. The specificity of this antibody was tested by SDS-PAGE and a Western blot. The Western blotting of junctional membrane proteins shows cross-reaction only with a band of an apparent molecular weight of 28 000. This band comigrated with pure MIP (data not shown).

NBD-PC-Mbs vesicles were incubated for 1 h at several dilutions of polyclonal anti-MIP (Figure 6). These vesicles were tested in the fluorescence assay. The anti-MIP serum was able to inhibit more than 90% of the initial quenching rate in a concentration-dependent manner. No effect was observed using preimmune serum as a control.

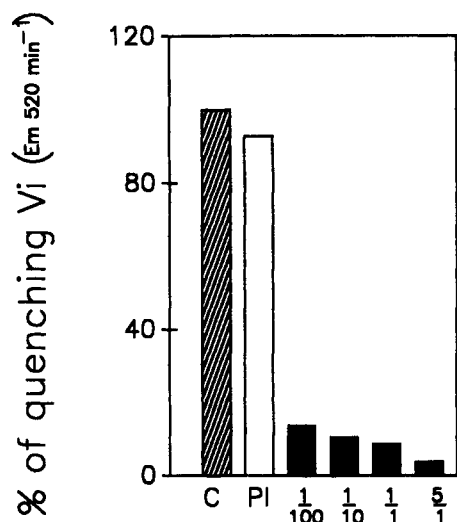


FIGURE 6: Effect of anti-MIP polyclonal antibody on PC-Mbs-induced vesicle aggregation. NBD-PC-Mbs vesicles ( $70 \mu\text{g}$  of protein) were incubated for 1 h at  $25^\circ\text{C}$  in reconstitution buffer with different anti-MIP serum dilutions (solid columns). As a control, NBD-PC-Mbs vesicles were also preincubated with preimmune serum (PI, open column). C (hatched column) was non-preincubated vesicles (normal assay conditions). After the preincubation,  $25 \mu\text{M}$  NBD-PC-Mbs was mixed with Rh-PS vesicles, and the initial rate of quenching was measured.

(ii) *HPLC-Purified MIP Reconstituted in LUV-Induced Aggregation.* In a second set of experiments, we purified MIP from solubilized junctional membrane proteins by HPLC (Ehring et al., 1992). The highly purified MIP was reconstituted into PC vesicles (NBD-PC-MIP). SDS-PAGE of these vesicles showed that MIP was successfully reconstituted into labeled vesicles (NBD-PC-MIP).

Figure 7 shows the emission time course at  $520 \text{ nm}$  of NBD-PC vesicles reconstituted with pure MIP. Immediately after the addition of Rh-PS vesicles (arrow), a rapid decline in the NBD emission occurs, reaching 65% (28, arbitrary units) of the initial fluorescence (80, arbitrary units). In this figure, faster kinetics can be observed compared with those obtained with total membrane protein reconstituted vesicles. The MIP-induced LUV aggregation was seen down to a molar ratio for MIP/lipid of as low as  $1/266$ . To prove that the LUV aggregation effect is a specific property of MIP, NBD-PC-MIP vesicles were previously incubated with anti-MIP serum as described above. The preincubation with anti-MIP antibody resulted in a high reduction in quenching (Figure 7).

As a control experiment, liposomes reconstituted with the remaining junctional membrane proteins, eluted as a broad peak during the HPLC purification of MIP (Ehring et al., 1992), did not aggregate with PS vesicles, showing that only MIP exhibits this property.

## DISCUSSION

It has been suggested that, in lens junctional membranes, MIP would principally function to promote cell adhesion between lens fiber cells (Zampighi et al., 1989). Starting from the primary structure based on cDNA cloning, Goring et al. (1984) proposed a putative transmembrane structure that shows three extracellular domains. None of them presents negatively charged amino acids. Two of them are formed by sequences of approximately 20 amino acids including arginine (112, 185, 196) and histidine (40, 122), which are both positively charged at pH's below their  $pK_a$ 's.

This prediction has led Zampighi et al. (1989) to postulate that junctions could be stabilized by electrostatic attractions

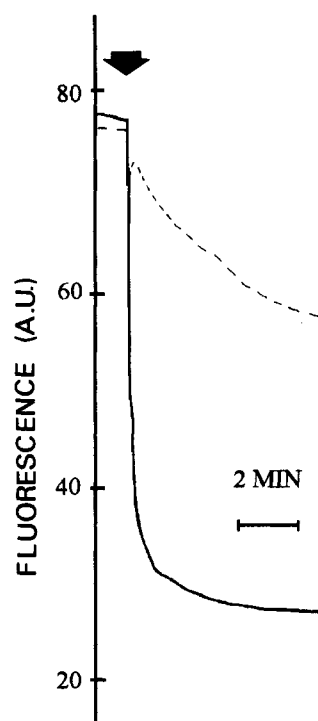


FIGURE 7: Specific aggregation of PC-MIP vesicles with PS vesicles detected by fluorescence assay: effect of anti-MIP serum. NBD-PC-MIP vesicles were preincubated in  $100 \text{ mM}$  NaCl and  $25 \text{ mM}$  Bis-Tris (pH 6) at  $25^\circ\text{C}$ . After 3 min, Rh-PS vesicles were added to the cuvette (arrow). The time course of NBD fluorescence emission at  $520 \text{ nm}$  was recorded. The solid line shows the aggregation between NBD-PC-MIP and Rh-PS vesicles. The dashed line shows the inhibition of the aggregation using NBD-PC-MIP vesicles preincubated with anti-MIP serum, as described for Figure 6 (serum dilution:  $1/100$ ).

between positively charged amino acids, located in the external domain of the MIP molecule, and negatively charged lipids on the opposing membrane (Zampighi et al., 1992). If we consider that MIP is arranged in a tetrameric structure, each MIP tetramer would contain a cluster of at least 20 positive charges. Such a charge density would cause strong electrostatic attraction, resulting in junctions having extremely narrow extracellular spaces.

The aim of our work was to test the possibility of MIP forming ionic interactions with negatively charged membranes. We chose PS large unilamellar liposomes because their properties have been thoroughly studied, and it is well-known that they do not associate with PC liposomes.

By measuring the NBD emission quenching at  $520 \text{ nm}$ , it was possible to demonstrate that only vesicles that were reconstituted in the presence of solubilized lens junction proteins or pure MIP were able to aggregate with PS vesicles. Moreover, using an absorbance assay, we also show that only vesicles that have MIP incorporated aggregate with PS vesicles.

This aggregation process is specifically dependent on the presence of MIP in one vesicle population. The evidence that supports this is as follows: First of all, proteolytic treatment with pronase inhibits the aggregation capability of LUV reconstituted with solubilized junction proteins with PS vesicles. Here, SDS-PAGE studies show that reconstituted MIP is the membrane protein most vulnerable to digestion by pronase (data not shown). Secondly, preincubation of LUV reconstituted with proteins solubilized from lens junctions with MIP polyclonal antibody also inhibits the aggregation. Thirdly, LUV reconstituted with pure MIP preincubated with anti-MIP serum also inhibits the vesicle aggregation. This inhibition was observed at a serum dilution of as low as  $1/600$ .

Furthermore, the presence of phosphatidylserine in the other population of vesicles is a prerequisite for LUV aggregation. PC-MIP vesicles aggregated only with PS vesicles. NBD-PC-Mbs vesicles mixed with Rh-PC or Rh-PC-Mbs vesicles did not show any effect on the NBD quenching emission. Also, no increase in absorbance at 350 nm was observed when these vesicle populations were mixed. Non-NBD-PC-MIP and Rh-PC-MIP vesicle aggregation was seen, demonstrating that this event was not due to MIP-MIP interactions.

Our results show that the aggregation is dependent on the presence of both MIP and PS. The liposomes thus aggregate through protein-lipid interactions, which are most likely electrostatic in nature. In principle, this interaction could be either hydrophobic or ionic. If PC-MIP vesicles interacted with PS vesicles through a hydrophobic domain, we would expect that PC-MIP vesicles would also aggregate with the reconstituted PC liposomes at measurable rates. In our assay conditions, PC vesicles did not aggregate with PC-MIP liposomes. Moreover, we did not observe lipid mixing between PC-MIP and PS liposomes. MIP causes adhesion of the vesicles, thereby bringing them close together, but does not cause lipid exchange. We interpret this result as evidence that MIP interaction with PS does not bring about a local destabilization of the PS bilayer through, for example, hydrophobic interactions.

The addition of  $Mg^{2+}$  or  $Ca^{2+}$  at pH 6.0 reduced the rate of aggregation of PC-MIP with PS vesicles; the binding of divalent cations to PS liposomes reduces their surface charge (McLaughlin et al., 1981), and this should in turn decrease the rate of interaction with PC-MIP vesicles, if this interaction is ionic. Therefore, we conclude that the interaction between reconstituted PC-MIP liposomes and negatively charged PS liposomes is primarily electrostatic in nature, suggesting that junctions composed of MIP may be stabilized by electrostatic forces. Because the tetrameric structure is retained even when the protein solubilizes in detergent micelles (Zampighi et al., unpublished experiments), and because PC-MIP large vesicles display tetragonal arrays identical to the MIP arrays demonstrated in freeze-fracture studies of isolated lens fiber junctions and whole lenses (Zampighi et al., 1990), in our experiments MIP should form crystalline arrays like those seen in native membranes, so that each array would have a positive charge cluster.

Electronic excitation energy can be transferred efficiently between the probes over distances of less than 10 nm (Duzgunes et al., 1987). Considering the high RET efficiency in our assays, we are led to presume that RET is taking place between probes on the internal sides of the bilayers as well as the external sides. As the average width of an artificial lipid bilayer is around 4.1 nm (McIntosh et al., 1984; Mason et al., 1981), the space between aggregated vesicles in our assay must be less than 2 nm. Therefore, the distance between adhered bilayers in our experiments should be similar to that previously found at lens fiber junctions (Zampighi et al., 1989).

The lens membrane lipid compositions of bovine, human, and other vertebrates are very much alike (Anderson et al., 1969; Broekhuysse 1969; Alcalá et al., 1982; Li et al., 1985); 10–14% of the membrane lipids are negatively charged phospholipids, phosphatidylserine being the most abundant (8–10% of total lipid) (Anderson et al., 1969; Broekhuysse, 1969; Lu-Ku et al., 1985). Considering these observations, we suggest that, in the lens fiber junction, MIP could be interacting electrostatically with phosphatidylserine, similar to what we have shown here. These results show the specific

aggregation between MIP and PS membranes. We conclude that this interaction is due to an electrostatic effect.

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