Interaction of the Sperm Adhesive Protein, Bindin, with Phospholipid Vesicles. II. Bindin Induces the Fusion of Mixed-Phase Vesicles That Contain Phosphatidylcholine and Phosphatidylserine In Vitro

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ABSTRACT Bindin from sea urchin sperm associates with gel-phase phospholipid bilayers (Glabe, C. G., 1985, J. Cell Biol., 100:794–799). Bindin also interacts with phospholipid vesicles containing both gel-phase and fluid-phase domains and thereby induces their aggregation. Association of bindin with vesicles containing gel-phase domains of dipalmitoylphosphatidylcholine (DPPC) and fluid-phase domains of brain phosphatidylserine (PS) was found to result in the fusion of the vesicles. After incubation with bindin, these mixed-phase vesicles were much larger as determined by gel filtration chromatography and electron microscopic observations of negatively stained samples. The average diameter of the vesicles after incubation was 190 ± 109 nm compared with 39 ± 20 nm for vesicles incubated in the absence of bindin. Resonance energy transfer studies also indicated that bindin induces the fusion of vesicle bilayers. Two fluorescent probes (NBD-PE and Rh-PE) were incorporated into the membrane of mixed-phase DPPC:PS vesicles at a density of 0.5 mol%, where efficient energy transfer occurs between the probes. The efficiency of energy transfer was proportional to the concentration of the fluorescence energy acceptor in the bilayer. The fluorescent vesicles were mixed with an excess of unlabeled target vesicles to quantify fusion. After bindin addition, there was a significant decrease in the efficiency of energy transfer compared with controls incubated in the absence of bindin. Although bindin induced the fusion of vesicles in the absence of calcium, the rate of fusion in the presence of 2 mM calcium was three-fourfold higher. In the presence of calcium, approximately half of the vesicles in the population had fused with another vesicle after incubation with bindin for 20 min. Bindin did not induce the fusion of gel-phase DPPC vesicles or mixed-phase vesicles of DPPC and dioleoylphosphatidylcholine, which suggests that the fusagenic activity of bindin requires specific phospholipids. Electron microscopic observations of DPPC:PS vesicles incubated in the presence of bindin suggest that the outer leaflets of bindin-aggregated vesicles are in close apposition. This is believed to be an important initial event for membrane fusion. These observations suggest that bindin may play a dual role in fertilization: Bindin mediates the attachment of sperm to glycoconjugate receptors of the egg surface and may also participate in the fusion of the sperm and egg plasma membranes.

Fusion of the sperm and egg plasma membranes is an important event of gamete interaction which is necessary to deliver the sperm nucleus into the egg cytoplasm (5). The sperm plasma membrane appears to be regionally specialized to perform this important role of membrane fusion in gamete interaction. In mammals, the site of sperm-egg fusion is restricted to an area of the sperm head known as the post-acrosomal region (31). In sea urchins, the plasma membrane...
that covers the acrosomal process appears to be specialized for fusion with the egg plasma membrane. This area of the sea urchin sperm plasma membrane arises from the former membrane of the acrosome granule after the exocytosis of this granule during the acrosome reaction. Fusion with the egg plasma membrane is restricted to this region of the sea urchin sperm membrane, which is also capable of fusing with other plasma membranes such as the sperm plasma membrane overlying the flagellum and mitochondrion (reference 4 and C. G. Glabe, unpublished observation). The acrosomal process of sea urchin sperm is coated with bindin, a 30,000-mol-wt polypeptide contained in the acrosomal granule, which is believed to function in the adhesion of sperm to the egg vitelline layer (9, 10, 12, 24). Immunohistochemical studies have shown that bindin is localized to the same restricted region of the sperm that fuses with the egg plasma membrane (18).

The membrane of the acrosomal process is functionally similar to the membrane of enveloped viruses. These viral membranes are also specialized for membrane fusion, which is required to transfer the viral genome into the cellular cytoplasm. The ability of the virus to fuse with cell membranes is due to the presence of specific viral proteins that appear to mediate this process and these fusogenic proteins are able to induce the fusion of phospholipid vesicles in vitro (29). In contrast to the extensive body of knowledge on virus fusion, almost nothing is known about the molecular basis of sperm–egg fusion. In the previous paper (8), I reported that bindin associates with phospholipid bilayers and preferentially interacts with gel-phase phospholipids. Here I report that bindin also associates with vesicles containing mixed-gel and fluid-phase domains. The association of bindin with vesicles containing dipalmitoylphosphatidylcholine (DPPC) and brain phosphatidylserine (PS) results in the fusion of the vesicles in vitro. These observations suggest that bindin may be responsible for the fusogenic properties of the sperm acrosomal process.

**MATERIALS AND METHODS**

**Gametes and Isolation of Bindin:** Preparation of gametes and isolation of bindin was performed as described in the preceding paper (8).

**Preparation of Lipid Vesicles:** Phospholipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) or Sigma Chemical Co. (St. Louis, MO). N-(nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and N-(tissue rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were from Avanti Polar Lipids, Inc. Phospholipid mixtures were dissolved in chloroform/methanol (2:1), dried under a stream of nitrogen gas, and lyophilized. 5 mg of lyophilized phospholipid was suspended in 1 ml of 0.54 M NaCl, 20 mM Tris, pH 8.0 (TBS) and sonicated using a standard microprobe (Heat Systems-Ultrasonics, Inc., Plainview, NY) in a 12 ml conical glass centrifuge tube under N2 at a power level of 50 W for 5 min. Temperature was maintained above the phase transition temperature of the lipid. The resulting vesicles were centrifuged at 1,000 g for 5 min to remove particulate material. Resonance energy transfer assays were performed as described by Strack et al. (23). Vesicles containing 0.5 mol% each of NBD-PE and Rh-PE (10 µl) were mixed with unlabeled vesicles (90 µl) in 0.7 ml TBS (500 µg total phospholipid). Aliquots of this mixture (100 µl) were diluted with 2 ml of TBS, and emission spectra were recorded between 460 and 640 nm while exciting at 450 nm using a Perkin-Elmer MFP-3 fluorometer (Perkin-Elmer Corp., Norwalk, CT). The extent of fusion was estimated from the fluorescence spectra by comparing the apparent initial and final molar ratios of fluorescent/nonfluorescent lipid of the probe vesicles. A standard curve was constructed to relate the ratio of nitrobenzoxadiazol (NBD)/rhodamine intensity to the concentration of fluorescent lipid in the bilayer within the range of 0.05–0.5 mol%. The resulting fusion index was expressed as the average number of nonfluorescent vesicles with which each fluorescent vesicle has fused. This fusion index depends on the assumption that the fluorescent and nonfluorescent vesicles are the same size, and this has been verified by electron microscopic observations of the vesicles. The fusion index is an average value for all of the vesicles in the population, but it seems likely that not all of the vesicles in the population fuse (see below). Therefore the fusion index may be higher for those vesicles in the population that actually fuse.

**Other Procedures:** Vesicle samples (0.7 ml) were fractionated on a Sepharose CL-4B column (1 x 60 cm) in TBS. Fractions of 1.5 ml were collected and the fluorescence of Rh-PE was determined (excitation, 535 nm; emission, 585). For electron microscopy of negatively stained samples, a drop of vesicle sample was applied to a newly glow-discharged, carbon-coated paraffin film on a 400-mesh microscope grid and incubated for 1 min. Excess samples was removed, a drop of 1% (wt/vol) potassium phosphotungstate, pH 6.5, was added, and the sample was stained for 30 s. Excess stain was removed and the sample was air dried and viewed in a Zeiss EM 952 electron microscope. Freeze-fracture samples of vesicles in TBS contained 25% glycerol and were frozen in liquid freon and fractured in a Balzers 400 freeze-fracture apparatus (Balzers, Hudson, NH). The fractured samples were shadowed with 1.3–2.0 nm of platinum at an angle of 25°, and 10 nm of carbon and the replicas were viewed in a Phillips 301 electron microscope operated at 100 kV.

**RESULTS**

As reported in the previous paper (8), bindin preferentially associates with gel-phase phospholipid vesicles, such as DPPC vesicles at 20°C. Bindin also associates with vesicles containing both gel- and fluid-phase domains. The detailed phase diagram available for the binary mixture of DPPC and brain PS indicates that for equimolar amounts of both lipids at 20°C, equivalent size domains of gel-phase DPPC (phase transition temperature \(T_m \approx 36°C\) in the mixture) and fluid-phase PS \(T_m = \approx 4°C\) exist in the membrane (22). Vesicles prepared from an equimolar mixture of DPPC and brain PS aggregate after addition of bindin (data not shown). Bindin does not interact with pure PS vesicles (K. Hong and C. Glabe, unpublished observation). This suggests that bindin aggregates mixed-phase vesicles interacting with the gel-phase DPPC domains.

After incubation with bindin, the mixed-phase DPPC:PS vesicles were found to be much larger. Most bindin-treated vesicles chromatographed in the void volume of Sepharose CL 4-B in contrast with vesicles incubated in the absence of bindin, which migrated in the included fraction (Fig. 1). However, these results do not distinguish between vesicles that are larger because they are aggregated and vesicles that have fused. Electron micrographs of negatively stained preparations indicated that after bindin addition, the average diameter of the vesicles was much larger: 190 ± 109 nm in the presence of bindin (Fig. 2a) as compared with 39 ± 20 nm for control samples incubated in the absence of bindin (Fig. 2b). The simplest explanation for this observation is that the larger vesicles are formed as a result of fusion of the smaller vesicles after bindin addition. If we assume that the surface area of the larger vesicles is the conserved product of the fusion of smaller vesicles, the large vesicles are the result of the fusion of an average of 22.5 of the small vesicles.

Resonance energy transfer studies also demonstrate that bindin induces the fusion of the vesicles. Several recent studies have shown that resonance energy transfer between NBD-PE as an energy donor and Rh-PE can be used to quantify membrane fusion (3, 15, 23). This method relies on the ability of NBD to efficiently excite rhodamine if the spatial separa-
Fractionation of the energy donor (NBD) and energy acceptor (rhodamine) is not great (23). A population of vesicles containing 0.5 mol% of both dyes (DPPC/PS/NBD-PE/Rh-PE, 49.5:49.5:0.5:0.5) showed efficient energy transfer between NBD and rhodamine (Fig. 3, a and a'). Most of the fluorescent emission was due to energy transfer from NBD to rhodamine (emission maximum, 585 nm). The amount of rhodamine fluorescence due to direct excitation at 450 nm is negligible (23). These vesicles were subsequently diluted with a ninefold excess of unlabeled vesicles for vesicle fusion assays. After incubation with bindin for 16 h, there was a significant decrease in the efficiency of energy transfer (Fig. 3, c and c') as compared with control vesicles incubated in the absence of bindin (Fig. 3, b and b'). This was apparent as an increase in the fluorescent emission of NBD (Fig. 3c) (emission maximum, 535 nm) along with a corresponding decrease in rhodamine fluorescence (Fig. 3 e'). The decrease in the efficiency of energy transfer is most likely caused by the equilibration of the dyes at a lower surface density owing to lateral diffusion after the dye-containing vesicles fuse with unlabeled vesicles. This sample was subsequently fractionated by gel filtration on Sepharose CL4-B. The largest vesicle fraction formed after incubation with bindin (Fig. 1, V0 fractions 22–23) was substantially fused as judged by resonance energy transfer (Fig. 3, d and d'). This spectra was similar to that of control vesicles disrupted with 0.1% Triton X-100 (Fig. 3, e and e') which eliminates energy transfer between the pair of fluorescent dyes (23). These data also suggest that different vesicle subpopulations fuse to different extents or perhaps some vesicles in the population do not fuse at all, in that the efficiency of energy transfer in this large subfraction (Fig. 3, d and d') was much less than that of the population as a whole (Fig. 3, c and c').

The kinetics of vesicle fusion was also determined by using the resonance energy transfer assay (Fig. 4). In the absence of calcium, the rate of bindin-induced fusion was significantly higher than the background rate in the absence of bindin. By 60 min of incubation, the fusion index was approximately 0.3, which suggests that on the average 30% of the fluorescent vesicles have fused with a nonfluorescent vesicle. (See Materials and Methods for the definition of fusion index.) After 16 h of incubation in the presence of bindin, the fusion index was 1.6 (Fig. 3, c and c'). In the presence of TBS containing 2 mM calcium, the rate of bindin-induced fusion was three- to fourfold higher. On the average, half of the vesicles had fused by 20 min in the presence of 2 mM calcium. At higher calcium concentrations, the rate of spontaneous fusion is much higher (data not shown; reference 6). The kinetics of vesicle fusion are slower than the kinetics for the aggregation of the vesicles by bindin, which is maximal by 5 to 10 min (8).
The ability of bindin to induce the fusion of other types of vesicles was partially investigated. Although DPPC vesicles are aggregated by bindin (8), they do not fuse. No decrease in energy transfer efficiency was observed for vesicles containing pure DPPC (Fig. 5, a and a' and b and b') or for mixed-phase vesicles containing equal amounts of DPPC and dioleoylphosphatidylcholine (DOPC) (Fig. 5, c and c' and d and d'). This result argues against the possibility that the decrease in energy transfer efficiency observed for DPPC:PS vesicles after bindin addition might be due to bindin-catalyzed exchange of fluorescent lipid probes between the vesicle populations. The rate of spontaneous exchange of the fluorescent probes in phospholipid vesicles alone is too low to measure (23).

Two factors appear to be important for the ability of membranes to fuse (19). (a) The outer leaflets of adjacent bilayers must overcome strong hydron and electrostatic forces that tend to keep them separated in order for the membranes of adjacent vesicles to closely appose each other. (b) Defects in the packing of the phospholipids appear to be

**Figure 3** Effect of bindin-induced vesicle fusion on resonance energy transfer between NBD-PE and Rh-PE. A 10-μl aliquot of DPPC:PS vesicles containing 0.5 mol% of both fluorescent lipids was diluted with a ninefold excess of unlabeled DPPC:PS vesicles in a total volume of 0.8 ml TBS. (a and a') Fluorescent peaks at 530 and 585 nm corresponding to NBD and rhodamine, respectively, in the absence of bindin at time zero. (b and b') Vesicle mixture incubated in the absence of bindin for 16 h. (c and c') Vesicles incubated in the presence of bindin for 16 h. (d and d') Bindin-treated vesicle fraction migrating in the void volume of Sepharose CL-4B column (Fig. 1). (e and e') Spectra obtained after solubilization of the phospholipid vesicles in 0.1% Triton X-100.

**Figure 4** Kinetics of bindin-induced vesicle fusion as determined by resonance energy transfer. Fluorescence spectra were recorded from a mixed population of fluorescent probe-containing and unlabeled vesicles at various times after incubation in the presence and absence of bindin as described in Fig. 3. The ratio of NBD to rhodamine intensity was used to estimate the change in the ratio of fluorescent to nonfluorescent lipid in the vesicle. The fusion index is the apparent average number of unlabeled vesicles that the population of fluorescent vesicles have fused with or, alternatively, the fraction of the population of labeled vesicles that have fused with at least one unlabeled vesicle if this value is less than one. [] Vesicles in TBS, incubated in the presence of bindin; [ ] vesicles in TBS, without bindin; [ ] vesicles in TBS containing 2 mM Ca²⁺, in the presence of bindin; [ ] vesicles in TBS containing 2 mM Ca²⁺, without bindin.

**Figure 5** Bindin addition has no effect on the efficiency of resonance energy transfer in DPPC and DPPC:DOPC (1:1) vesicles. (a and a') Spectra obtained for DPPC vesicles containing 0.5 mol% each of NBD-PE and Rh-PE in the absence of bindin. (b and b') Fluorescence peaks at 530 and 585 nm corresponding to the emission of NBD and rhodamine, respectively, after 16-h incubation in the presence of bindin. (c and c') Spectra obtained from DPPC:DOPC vesicles incubated in the absence of bindin for 16 h. (d and d') Spectra from vesicles incubated in the presence of 50 μg of bindin for 16 h. There is no change in the efficiency of energy transfer for DPPC vesicles or DPPC:DOPC vesicles after incubation with bindin, indicating that the vesicles do not fuse.
necessary to initiate an intermediate structure in which the outer leaflets of adjacent membranes become continuous. This intermediate may include an inverted micelle or hexagonal phase (19) although there are several model membrane systems in which fusion can be induced without apparently undergoing a hexagonal phase (2, 6). Bindin appears to participate in at least the first step of bringing the bilayers into close apposition. Negatively stained preparations reveal that the adjacent surfaces of vesicles appear to be in contact and the boundary between vesicles often appears flattened at the areas of contact (Fig. 6). Thus, the area of vesicle surface involved in contact with an adjacent vesicle is maximized. This negatively stained image must be interpreted with caution as the metal ions used for negative staining (phosphotungstate) have been shown to aggregate phosphatidylcholine vesicles (17). Although the control PS:DPPC vesicles do not appear to be aggregated by the negative stain in the absence of bindin (Fig. 2b), freeze fracture was also performed on this sample. Freeze-fracture images of DPPC:PS vesicles after bindin treatment also revealed that the outer leaflets of many of the aggregated vesicles were apparently in close apposition (Fig. 7), although flattened areas of contact between vesicles were not apparent. In addition, no intramembranous particles were observed in the fracture faces of the vesicles after incubation with bindin.

DISCUSSION

The results of this study indicate the interaction of bindin with sonicated vesicles of mixed DPPC and brain PS results in the fusion of the vesicles. That the vesicles are much larger after bindin addition, as judged by the chromatographic behavior of vesicles on Sepharose CL 4-B (Fig. 1) and observations of negatively stained vesicle preparations (Fig. 2), is evidence for this interpretation. After incubation in the presence of bindin, the average diameter of the vesicles was approximately fivefold larger than vesicles incubated in the absence of bindin. Resonance energy transfer studies also

**Figure 6** Electron micrograph of negatively stained DPPC:PS vesicles 16 h after incubation with bindin. Vesicle samples were diluted 1:10 with TBS and applied to a carbon- and parlodion-coated grid. Arrows point to flattened regions of the vesicle surface that are in contact with the surface of an adjacent vesicle. **x** 86,850.

**Figure 7** Freeze-fracture electron micrograph of DPPC:PS vesicles 16 h after bindin addition. Arrows point to regions of apparent close apposition of adjacent vesicles. **x** 64,200.
support the interpretation that the vesicles fuse after bindin addition (Fig. 3). In the absence of bindin, efficient energy transfer from NBD to rhodamine was observed in vesicles containing 0.5 mol% fluorescent lipids diluted with a ninefold excess of unlabeled vesicles. After incubation with bindin, the efficiency of energy transfer in the vesicle population was greatly reduced. This is consistent with the interpretation that the decrease in energy transfer efficiency after bindin addition is due to a decrease in the surface density of the fluorescent dyes by lateral diffusion in the plane of the membrane after fusion of the labeled vesicles with excess unlabeled vesicles. In the absence of bindin, there was only a slight decrease in the efficiency of energy transfer indicating that the vesicles do not spontaneously fuse at a high rate.

I have also partially investigated the lipid specificity of bindin-induced vesicle fusion. Bindin does not induce the fusion of vesicles that it does not aggregate, such as vesicles containing only brain PS (K. Hong and C. Glabe, unpublished), but not all of the vesicles that are aggregated by bindin fuse. Pure DPPC vesicles were aggregated by bindin but did not fuse (Fig. 5). This suggests that expression of the fusagenic activity of bindin may require the presence of phosphatidylserine since vesicles containing DPPC and DOPC can be induced to fuse with other fusagenic proteins such as concanavalin A (26), tubulin (14, 15), tobacco mosaic virus (1), and clathrin (3). Other fusagenic proteins require specific lipids. Synexin is capable of fusing brain PS vesicles, but not vesicles that contain both DPPC and PS (13). Fusion of Semliki Forest virus with target vesicles requires cholesterol and is optimal at cholesterol to phospholipid ratios of 0.5 or higher, but phosphatidylycholine–cholesterol vesicles do not serve as efficient target membranes for virus fusion (27). The fusogenic activity of the F protein of Sendai virus is relatively insensitive to the phospholipid composition of the membrane (reviewed in reference 29). The fusogenic activity of influenza virus hemagglutinin is also relatively insensitive to phospholipid composition (16) although fusion is reduced when phosphatidylyethanolamine is not present (28). Thus, the lipid specificity for bindin appears to be different than other fusagenic proteins for which the lipid specificity has been investigated. Further studies of the lipid specificity for bindin-induced membrane fusion are currently under way.

One way in which bindin might facilitate membrane fusion during fertilization is by mediating the close apposition of the outer leaflets of adjacent plasma membranes of sperm and egg. Bindin aggregates lipid vesicles in vitro, and electron micrographs of aggregated vesicles suggest that their surfaces are in close contact. Association of bindin with the bilayer may also alter the packing of the phospholipids which is important for the rearrangement of the outer leaflet of the bilayer which is thought to be a necessary intermediate in the fusion process. It is not yet clear whether the mixed fluid- and gel-phase physical properties of DPPC:PS vesicles (22) are important for the ability of these vesicles to fuse after bindin addition. Mixed-phase domain structure alone is apparently not sufficient for fusion since mixed-phase vesicles of DPPC:DOPC did not fuse upon bindin addition. Defects in the packing of membrane phospholipids are also believed to occur at the boundary between gel and fluid lipid domains (21). Recent studies suggest that the plasma membrane of unfertilized sea urchin eggs contains both gel and fluid lipid domains (30).

The ability of bindin to induce the fusion of some types of phospholipid vesicles in vitro may be related to the fusagenic activity of the sperm acrosome process. Bindin co-localizes with this area of the sperm plasma membrane, which appears to be specialized for membrane fusion. If bindin does participate in the fusion of sperm and egg plasma membranes, then bindin would play two roles in fertilization: adhesion of sperm to the egg surface and fusion of plasma membranes. This situation is functionally similar to the roles played by influenza virus hemagglutinin during viral infection (reviewed in reference 29). The influenza virus hemagglutinin functions in the adhesion of the virus to the cell surface by binding sialic acid residues of glycolipids and glycoproteins and also induces the fusion of membranes by interacting with phospholipid bilayers. Bindin is believed to mediate the adhesion of sperm to the egg surface by interacting with sulfated fucose-containing glycoconjugates of the egg vitelline layer (11, 20). The cell surface binding activities of influenza virus hemagglutinin and bindin are both expressed as a lectin-like ability to agglutinate erythrocytes (9). Bindin and influenza hemagglutinin are also structurally similar in the sense that their amino terminal polypeptide sequences are rich in hydrophobic residues. Bindin from Strongylocentrotus purpuratus contains a segment of 19 hydrophobic amino acids extending between residues 27 and 45 (25). Influenza viruses contain a 28 amino acid sequence at the N-terminus containing predominantly hydrophobic amino acids (7). This region is believed to function in the fusion of the viral membrane with the cellular plasma membrane (29). Although evidence has been presented that the influenza virus HA mediates the fusion between viral and cell membranes (29), this remains to be established for sea urchin sperm bindin.

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