

# Interaction of the Sperm Adhesive Protein, Bindin, with Phospholipid Vesicles. I. Specific Association of Bindin with Gel-Phase Phospholipid Vesicles

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**ABSTRACT** Bindin is a 30,000-mol-wt protein of sea urchin sperm that is responsible for the specific adhesion of the sperm acrosomal process to the vitelline layer covering the egg plasma membrane during fertilization. Sulfated glycoconjugates are believed to be the egg surface receptors for bindin, but the mechanism by which bindin associates with the sperm acrosomal membrane is unknown. Here I report that bindin specifically associates with phospholipid vesicles in vitro. Interaction of the bindin polypeptide with liposomes was found to cause an increase in the density of the liposomes and induce the aggregation of the vesicles. A novel property of this association of bindin with membranes was that it required phospholipids in a gel phase. The interaction of bindin with liposomes was greatly reduced at temperatures above the phase transition temperature. The interaction of bindin with gel-phase vesicles appeared to be reversible, since the aggregated vesicles disassembled as the temperature was raised above the phase transition temperature. Association of bindin with the bilayer did not alter the accessibility of the polypeptide to cleavage by trypsin, which suggests that most of the polypeptide chain remains exposed at the surface of the membrane.

Bindin is a 30,000-mol-wt protein that is contained in the acrosomal granule of sea urchin sperm (21). The granule undergoes exocytosis upon contact of the sperm with the egg surface, and it is the persistent contents of the granule that form a morphologically recognizable bond between the membrane of the sperm acrosomal process and the vitelline layer covering the egg plasma membrane (19). The evidence that supports the role of bindin in mediating this adhesion includes the following observations: Bindin particles (isolated acrosome granules) agglutinate unfertilized eggs (21) in a species-specific fashion (7, 9). Fertilized or trypsin-treated eggs, which do not bind sperm, are not agglutinated by bindin (21). Monospecific antibody to bindin localizes the bindin polypeptide to the acrosomal process of acrosome-reacted sperm and the site of the sperm-egg adhesive bond (15). This adhesive contact, 0.25  $\mu\text{m}$  diam, is sufficient to immobilize the sperm with an actively beating flagellum 50- $\mu\text{m}$  long. In order to mediate this seemingly tenacious adhesion, bindin must interact with components of the egg surface and also remain associated with the membrane of the acrosomal process. Sulfated glycoconjugates of the egg vitelline layer are believed

to function as receptors in the interaction of bindin with the egg (6, 8, 17), but it is not known how the bindin associates with the sperm acrosomal membrane. I have investigated the interaction of bindin with phospholipid vesicles in vitro. Bindin was found to associate directly with phospholipid bilayers in a gel state, but not with membranes above the major phospholipid phase transition temperature ( $t_m$ ).<sup>1</sup> This property of bindin may be instrumental for maintaining the association of bindin with the acrosomal process during sperm adhesion.

## MATERIALS AND METHODS

*Gametes and Isolation of Bindin:* Gametes of *Strongylocentrotus purpuratus* were obtained by pouring 0.5 M KCl into opened body cavities.

<sup>1</sup>*Abbreviations used in this paper:* DiI, 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; TBS, 0.54 M NaCl, 50 mM Tris, pH 8.0;  $t_m$ , phase transition temperature.

Bindin was isolated and purified as previously described (20, 21). Bindin was stored frozen at  $-20^{\circ}\text{C}$  in 0.54 M NaCl, 50 mM Tris, pH 8.0 (TBS) and sonicated at 10 W for 15 s before use. Bindin was iodinated as described (4).  $\text{Na}^{125}\text{I}$  was obtained from New England Nuclear (Boston, MA).

**Preparation of Lipid Vesicles:** Lipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) or Sigma Chemical Co. (St. Louis, MO). 5 mg of lyophilized phospholipid was suspended in 1 ml of buffer. TBS was used for phosphatidylcholine and 0.15 M NaCl, 20 mM Tris, pH 8.0, was used for phosphatidylglycerol to avoid the high spontaneous rate of aggregation that occurs above 0.25 M NaCl. The lipid dispersion was sonicated using a standard microprobe (Heat Systems-Ultrasonics, Inc., Plainview, NY) in a 12-ml conical glass centrifuge tube under  $\text{N}_2$  gas at a power level of 50 W for 15 min. Temperature was maintained above the phase transition temperature of the lipid during vesicle preparation. The resulting vesicles were centrifuged at 1,000 *g* for 5 min to remove particulate titanium from the sonicator probe. Electron microscopic observations of negative-stained preparations of the vesicles indicate that they are predominately unilamellar with a mean diameter of  $49 \pm 16$  nm and a size range of from 28 to 120 nm. Fluorescent liposomes were prepared by sonicating a mixture of 5 ml of phospholipid and 5  $\mu\text{g}$  of 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Junction City, OR) as described above. DiI fluorescence was quantified by exciting at 515 nm and measuring emission at 565 nm.

**Sucrose Density Gradient Centrifugation:** Samples for photographs were centrifuged on 15-ml sucrose step gradients. Step gradients consisted of 1 ml 60% (wt/wt) sucrose in TBS, 2 ml 30% sucrose in TBS, 5 ml 15% sucrose in TBS, and 6 ml 5% sucrose in TBS. Samples (0.5 ml) were layered on top of the gradient and centrifuged in a Beckman SW 40 rotor (Beckman Instruments, Inc., Palo Alto, CA), 30,000 rpm (relative centrifugal force<sub>max</sub> = 160,000 *g*) at  $20^{\circ}\text{C}$  for 14–24 h. After centrifugation, the tubes were photographed against a black background with illumination from the top. All other vesicle samples (150  $\mu\text{l}$ ) were centrifuged on 5-ml linear gradients of 5–15% (wt/wt) sucrose in TBS in a Beckman SW 50.1 rotor at 30,000 rpm (relative centrifugal force<sub>max</sub> = 108,000 *g*) for 22 h. After centrifugation, the gradients were fractionated and the DiI fluorescence and the amount of  $^{125}\text{I}$ -bindin were determined for each fraction.

**Liposome Aggregation:** Aggregation of liposomes was measured as an increase in turbidity. A 0.1-ml aliquot of liposomes was added to a cuvette containing 0.7 ml buffer. Bindin was added to the suspension and the absorbance at 400 nm was recorded. The buffers used for determining the pH dependence of aggregation contained 0.54 M NaCl, 50 mM sodium acetate, pH 4.5–5.5; 50 mM sodium phosphate, pH 5.5–7.5; 50 mM Tris, pH 7–9. No significant difference in the extent of turbidity increase was detected for the different buffers used in pH regions where they overlap. Buffers used to determine the ionic strength dependence of aggregation contained 20 mM Tris, pH 8, and 0–2 M NaCl. For experiments on the temperature dependence of disaggregation of vesicles, 100  $\mu\text{l}$  of dimyristoylphosphatidylcholine (DMPC)

vesicles (500  $\mu\text{g}$ ) were mixed in a cuvette with 1 ml of TBS and aggregated by incubation with 25  $\mu\text{g}$  of bindin for 10 min at  $18^{\circ}\text{C}$ . Absorbance at 400 nm was recorded as the temperature was increased at a rate of  $2^{\circ}\text{C}/\text{min}$ . Temperature in the cuvette was monitored with a Bausch & Lomb Thermindicator (Bausch & Lomb Inc., Rochester, NY). The reference cell of the spectrophotometer contained vesicles and buffer in the absence of bindin.

**Other Procedures:**  $^{125}\text{I}$ -bindin samples were digested with 25  $\mu\text{g}/\text{ml}$  trypsin at  $20^{\circ}\text{C}$  for 30 min. After being boiled to stop digestion, the samples were processed for SDS PAGE. Electrophoresis was performed by the method of Laemmli (13) on 15% polyacrylamide separating gels. After electrophoresis, the unfixed gel was dried with a slab gel dryer (Hoefer Scientific Instruments, San Francisco, CA) and autoradiography was performed at  $-70^{\circ}\text{C}$  using Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

## RESULTS

### Association of Bindin with Lipid Vesicles

Addition of bindin to dipalmitoylphosphatidylcholine (DPPC) vesicles resulted in an increase in the density of the vesicles as revealed by centrifugation in sucrose gradients at  $20^{\circ}\text{C}$  (Fig. 1). In the absence of bindin the vesicles did not enter the gradient, indicating that they have a density of  $<1.066$  g/cc (Fig. 1a). After sonication in the presence of bindin, the vesicles banded at a density of between 1.090 and 1.097 g/cc (Fig. 1b). In aqueous solutions bindin was in equilibrium between 100,000 *g* soluble bindin and an insoluble, particulate fraction of bindin (6). Bindin particles, in the absence of phospholipid vesicles, pelleted at the bottom of the gradient (Fig. 1c). Other proteins such as BSA and ovalbumin had no effect on the sedimentation behavior of the vesicles (data not shown). Sonication was not required for the association of bindin with the vesicles (Fig. 2a). Incubation of  $^{125}\text{I}$ -labeled

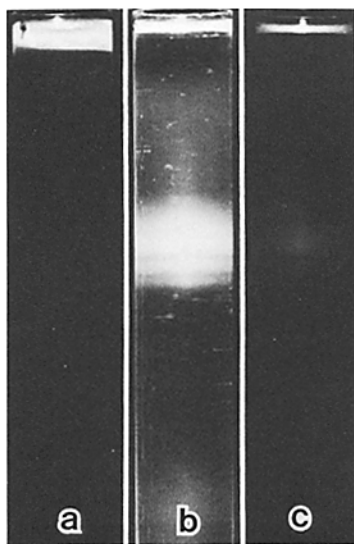


FIGURE 1 Sucrose density centrifugation of DPPC vesicles and bindin. (a) Control containing 0.5 ml DPPC vesicles (2.5 mg lipid) alone. The vesicles remain at the top of the gradient. (b) Vesicles sonicated in the presence of 200  $\mu\text{g}$  of bindin. (c) Control containing 200  $\mu\text{g}$  of bindin in the absence of vesicles. The bindin particles pellet at the bottom of the tube.

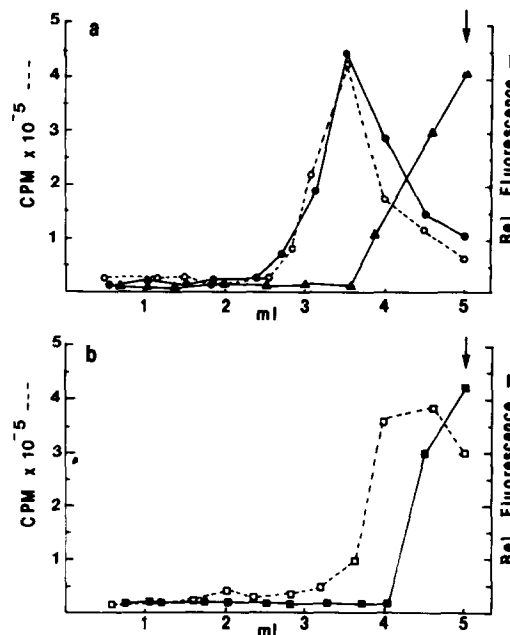


FIGURE 2 Sucrose density gradient sedimentation of  $^{125}\text{I}$ -bindin and DiI-containing phosphatidylcholine vesicles. (a) 25  $\mu\text{g}$  of  $^{125}\text{I}$ -bindin ( $8 \times 10^4$  cpm/ $\mu\text{g}$ ) was incubated with 100  $\mu\text{l}$  of DPPC (500  $\mu\text{g}$  lipid) vesicles for 20 min and then centrifuged on a sucrose gradient.  $\circ$ ,  $^{125}\text{I}$ -bindin.  $\bullet$ , DiI fluorescence of vesicles incubated with bindin. The bindin and the vesicles co-sediment. In the absence of bindin, the vesicles remain at the top of the gradient.  $\blacktriangle$ , DiI fluorescence of DPPC vesicles in the absence of bindin. (b) 25  $\mu\text{g}$  of  $^{125}\text{I}$ -bindin was incubated with 100  $\mu\text{l}$  of dioleoylphosphatidylcholine vesicles (500  $\mu\text{g}$  lipid) as described above and then centrifuged on a sucrose gradient.  $\square$ ,  $^{125}\text{I}$ -bindin.  $\blacksquare$ , DiI fluorescence. The bindin and the vesicles do not co-sediment. Arrows indicate the top of the gradients.

bindin with DPPC vesicles demonstrated that nearly all of the added bindin co-migrated with the vesicles at this higher density position in the gradient (Fig. 2a). Under the same conditions, bindin did not associate with dioleoylphosphatidylcholine ( $t_m = -22^\circ\text{C}$ ) vesicles (Fig. 2b). The dioleoylphosphatidylcholine vesicles and the soluble  $^{125}\text{I}$ -bindin remained at the top of the gradient.

### Bindin-induced Aggregation of Lipid Vesicles

The interaction of bindin with lipid vesicles also resulted in an increase in turbidity at 400 nm (Fig. 3). Electron microscopic observations of these vesicles after negative staining suggested that this increase in turbidity is due to aggregation of the vesicles (see the accompanying paper, [5]). The association of bindin with DPPC vesicles did not require  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  since this interaction occurred equally well in 0.54 M NaCl and 50 mM Tris, pH 8 (TBS), and in sea water and 50 mM Tris, pH 8, which contains 11 mM  $\text{Ca}^{++}$  and 55 mM  $\text{Mg}^{++}$  (Fig. 3). The bindin-induced aggregation of the vesicles was maximal by 5–15 min and was stable for at least 24 h (data not shown). The extent of the aggregation was dependent on the concentration of bindin, and aggregation was optimal at lipid/protein mass ratios of approximately 5:1 (200 mol/mol) (Fig. 4).

The effect of NaCl concentration and pH on the aggregation of DPPC liposomes was also investigated. The extent of bindin-induced vesicle aggregation increased linearly with increasing NaCl concentration up to a maximum at 1 M NaCl (Fig. 5a). In the absence of NaCl (20 mM Tris), the extent of vesicle aggregation was <10% of the maximal extent of aggregation. The pH optimum for the bindin-induced aggregation of DPPC vesicles was ~6 (Fig. 5b). Over the entire pH range of 4.5–9.0, there was less than a threefold change in the extent of vesicle aggregation.

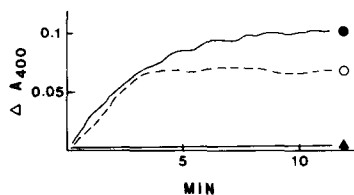


FIGURE 3 Kinetics of the bindin-induced aggregation of DPPC vesicles. 25  $\mu\text{g}$  of bindin was added to a suspension of 100  $\mu\text{l}$  of vesicles (500  $\mu\text{g}$  lipid) in 0.7 ml sea water, 50 mM Tris pH 8.0, O, or in 0.7 ml TBS, ●. The extent of aggregation is maximal by 5–10 min. There is no change in the turbidity of the vesicles in the absence of bindin, ▲.

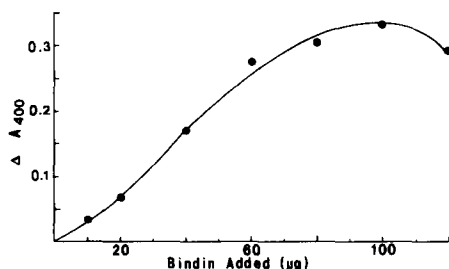


FIGURE 4 Concentration dependence of bindin-induced liposome aggregation. Increasing amounts of bindin were added to a suspension of 100  $\mu\text{l}$  DPPC vesicles in 0.7 ml TBS, and the maximum extent of turbidity increase was determined at 400 nm.

### Specificity of Bindin for Gel State Phospholipids

I also investigated the effect of varying the fatty acid chain and head group type of the lipid on the interaction of bindin with vesicles. Table I summarizes the results obtained after bindin addition to different types of phospholipid vesicles. At  $20^\circ\text{C}$ , only vesicles containing gel-phase lipids (distearoyl-, dipalmitoyl-, and dimyristoylphosphatidylcholine) exhibited an interaction with bindin as judged by both a density shift on sucrose gradients and an increase in turbidity at 400 nm after bindin addition (Fig. 6, a and b). By the same criteria, bindin did not aggregate vesicles containing fluid-phase lipids at  $20^\circ\text{C}$  (dilauroyl-, dioleoyl-, and dilinoleoylphosphatidylcholine) (Fig. 6a). When the experiment was performed at  $26^\circ\text{C}$ , the bindin-induced aggregation of DMPC ( $t_m = 23^\circ\text{C}$ ) vesicles was greatly reduced although it still aggregated DPPC ( $t_m = 41^\circ\text{C}$ ) vesicles, indicating that the bindin is not denatured at

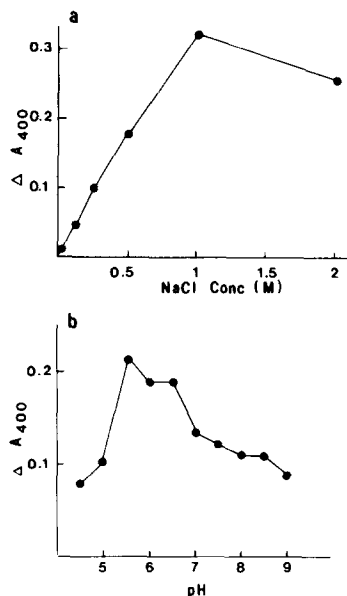


FIGURE 5 Effect of NaCl concentration and pH on the interaction of bindin with DPPC vesicles. (a) 50  $\mu\text{g}$  of bindin was added to a suspension of 100  $\mu\text{l}$  DPPC vesicles in 0.7 ml 20 mM Tris, pH 8.0, containing various NaCl concentrations in the range of 0–2 M. The extent of vesicle aggregation was determined by the maximal increase in turbidity at 400 nm. (b) The effect of pH on the interaction of bindin with DPPC vesicles was determined by adding 50  $\mu\text{g}$  of bindin to a 100- $\mu\text{l}$  suspension of DPPC vesicles in 0.7 ml buffer, pH 4.5–9, containing 0.54 M NaCl.

TABLE I

Effect of Fatty Acid Chain and Head Group Composition on Interaction of Bindin with Phospholipid Vesicles at Different Temperatures

Lipid	$t_m$	Interaction with bindin?	
		$20^\circ\text{C}$	$26^\circ\text{C}$
Dilinoleoylphosphatidylcholine	<0	–	–
Dioleoylphosphatidylcholine	–22	–	–
Dilauroylphosphatidylcholine	0	–	–
Dimyristoylphosphatidylcholine	23	+	–
Dipalmitoylphosphatidylcholine	41	+	+
Distearoylphosphatidylcholine	58	+	+
Dimyristoylphosphatidylglycerol	23	+	–
Dipalmitoylphosphatidylglycerol	41	+	+

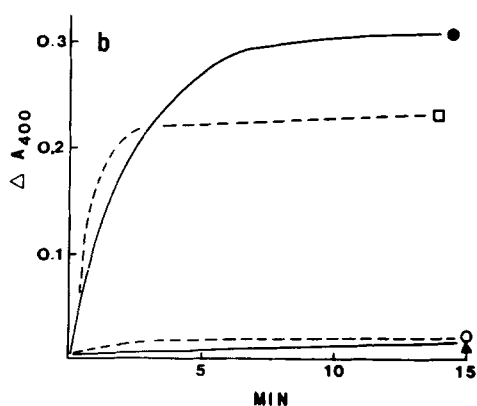
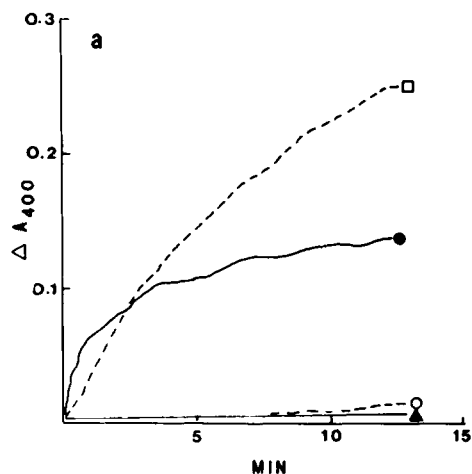


FIGURE 6 Bindin-induced aggregation of different types of phospholipid vesicles at 20° or 26°C. 50  $\mu\text{g}$  of bindin was added to a suspension of 100  $\mu\text{l}$  of vesicles in 0.7 ml TBS. (a)  $\blacktriangle$ , Dioleoyl-, dilauroyl-, and dilinoleoylphosphatidylcholine at 20° C.  $\bullet$ , DMPC, 20°C.  $\circ$ , DMPC, phosphatidylcholine, 26°C.  $\square$ , DPPC, 26°C. (b) Bindin-induced aggregation of DMPG and DPPG vesicles at 20° and 26°C.  $\blacktriangle$ , DMPG vesicles in the absence of bindin, 20°C.  $\bullet$ , DMPG vesicles in the presence of bindin at 20°C.  $\circ$ , DMPG vesicles in the presence of bindin at 26°C.  $\square$ , DPPG vesicles in the presence of bindin at 26°C.

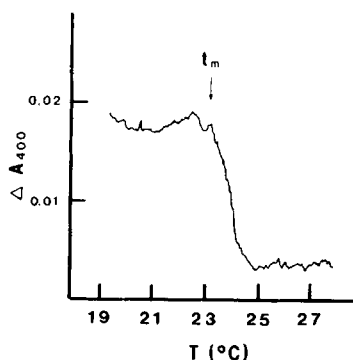


FIGURE 7 Temperature dependence of the disaggregation of bindin-aggregated DMPC vesicles. DMPC vesicles (150  $\mu\text{l}$  in 1 ml TBS) were aggregated by incubation in a 1.5-ml cuvette with 25  $\mu\text{g}$  bindin for 10 min. After incubation, the temperature was raised at a rate of 2°C/min and the absorbance at 400 nm was recorded. As the temperature of the vesicles reached the major phase transition temperature, ( $t_m$  for DMPC = 23°C), a marked decrease in the absorbance of the sample was observed.

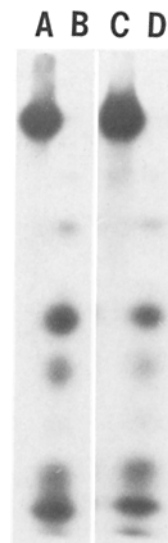


FIGURE 8 Autoradiogram of 15% polyacrylamide SDS slab gel of bindin and trypsin digestion products of bindin. (A)  $^{125}\text{I}$ -bindin. (B) Trypsin digestion products of  $^{125}\text{I}$ -bindin in the absence of vesicles. (C) DPPC vesicle-associated bindin. (D) Trypsin digestion products of vesicle-associated  $^{125}\text{I}$ -bindin.

26°C (Fig. 6a). Bindin also induced the aggregation of dimyristoylphosphatidylglycerol (DMPG,  $t_m = 23^\circ\text{C}$ ) and dipalmitoylphosphatidylglycerol (DPPG,  $t_m = 41^\circ\text{C}$ ) vesicles at 20°C, indicating that the association of bindin with the membrane does not depend on the head group type of the phospholipid (Fig. 6b). Again, at 26°C, the aggregation of fluid-phase DMPG vesicles was greatly reduced, in contrast to the gel-phase DPPG vesicles. These results suggest that the association of bindin with the phospholipid bilayer is regulated by the physical state of the bilayer. In addition, it appears that the association of bindin with DMPC vesicles is reversible at temperatures above the major phase transition temperature (Fig. 7). DMPC vesicles were aggregated by addition of bindin at 18°C and heated at a rate of 2°C/min to 26°C. As the temperature reached the major phase temperature for DMPC (23°C), the vesicles disaggregated as measured by a decrease in turbidity, which suggests that the association of bindin with gel-phase phospholipids may be reversible.

#### Accessibility of Vesicle-associated Bindin to Proteolysis

The effect of vesicle association on the accessibility of the bindin polypeptide to proteolysis by trypsin was investigated. All of the vesicle-associated bindin was digested by trypsin, indicating that the bindin is not encapsulated by the liposome (Fig. 8). The pattern of trypsin cleavage fragments for vesicle-associated bindin was identical to the pattern obtained for bindin in the absence of vesicles, suggesting that all of the trypsin cleavage sites are exposed at the surface of the vesicle and not integrated within the bilayer. This does not rule out the possibility that the hydrophobic N-terminus of the bindin polypeptide might interact with the hydrocarbon chain region of the fatty acids since the first available trypsin cleavage site occurs at residue number 70 (22).

#### DISCUSSION

These results demonstrate that bindin is an amphipotent protein (12), which is defined as a protein that can exist in a

water-soluble or lipid-associated state. Other examples of amphipotent proteins include tubulin (2, 10, 12), apolipoproteins of serum lipoproteins (14), concanavalin A (23), M13 bacteriophage coat protein (24), and components of the serum complement system (16). The present findings suggest that bindin associates only with membranes containing lipids in a gel-phase physical state. In contrast, the apolipoprotein Apo C-III prefers fluid-state lipids (14). Tubulin interacts only at the gel-fluid phase transition temperature (10). Concanavalin A interacts with DPPC vesicles at temperatures above and below the phase transition temperature (23), but the rate of vesicle fusion induced by concanavalin A exhibits a strong maximum at the phase transition temperature. The M13 bacteriophage coat protein is incorporated into vesicles upon detergent dilution at temperatures well below the phase transition, but it is correctly inserted with an orientation spanning the bilayer only when the vesicles are assembled at the lipid phase transition temperature (25). Thus bindin may be an example of an amphipotent membrane protein that specifically associates with gel-phase domains of membranes. This suggests a molecular mechanism whereby changes in the physical state of the membrane might regulate the biological function of the membrane by controlling the association of specific proteins. Different phase domains of phospholipids have been postulated to exist in biological membranes (1, 11, 18, 26). Since bindin preferentially interacts with gel phase lipids, it may be an important tool for studying phase domains in biological membranes.

It is not yet clear how the bindin polypeptide interacts with the phospholipid bilayer or how lipid structure below the phase transition apparently regulates this interaction. Polar interactions appear to play a relatively minor role in the association of bindin with lipid vesicles. Bindin interacts with both phosphatidylcholine and phosphatidylglycerol vesicles which suggests that the charge of the polar head group is not critical for this interaction. The interaction of bindin and lipid vesicles is optimal at relatively high ionic strength and displays a broad pH optimum in the range of 4.5–9.0. The finding that bindin only interacts with gel-phase lipid vesicles also argues against the role of electrostatic interactions with the polar head groups in the association of the bindin polypeptide with the bilayer. It seems unlikely that the fluid state of the bilayer would have any effect on the ionic interactions of the head groups. It seems most likely that the hydrophobic N-terminal region of the bindin polypeptide may interact hydrophobically with the hydrocarbon region of the fatty acid moieties of the lipid bilayer. The bindin polypeptide contains only 13 noncharged polar residues in the first 56 amino acids. On the basis of this N-terminal sequence analysis, it was proposed that the segment of the polypeptide extending between residue 27 and 52 may be associated with the hydrophobic region of the bilayer (22). This section contains two noncharged, polar amino acid residues and a continuous segment of 15 hydrophobic residues. It also seems likely that the remainder of the bindin polypeptide is exposed at the surface of the membrane, because all of the trypsin cleavage sites remain accessible to trypsin digestion when bindin is associated with the membrane.

Since bindin functions in the adhesion of sperm to the egg during fertilization, there must be some mechanism for its stable association with the acrosomal membrane of the sperm. Therefore, it seems reasonable that bindin associates directly with membranes. The other alternative would be for bindin

to associate with a "receptor" in the membrane of the acrosomal process. Another potential implication of the interaction of bindin with membranes is that bindin may function in the fusion of sperm and egg plasma membranes. There is some circumstantial evidence in support of this notion. The bindin-coated acrosomal process is specialized for fusion with membranes. Only this region fuses with the egg plasma membrane and it is also capable of fusing with other types of membranes that do not normally fuse (3). Since this entire region of the sperm is coated with bindin (15), it seems reasonable that the bindin is either instrumental in the fusogenicity of this region or impedes this process. One way in which bindin could conceivably promote membrane fusion is by associating with both the sperm and egg plasma membrane thus bringing them in closer apposition than they would normally come due to hydration force repulsion. Overcoming this repulsion force is a critical event in membrane fusion (24). The ability of bindin to aggregate phospholipid vesicles may be a manifestation of the potential of bindin to interact with sperm and egg plasma membranes *in vivo*. In the accompanying paper (5), I examine the potential role of bindin in the fusion of sperm and egg plasma membranes and present evidence that suggests that bindin promotes the fusion of phospholipid vesicles *in vitro*.

I thank Ms. Mary Yacuzzi for technical assistance and Drs. David Deamer, Keelung Hong, Kenneth Jacobson, Richard Pagano, Judith White, and David Wolf for helpful discussions. The initial observation on the interaction of bindin with DPPC liposomes was made while I was a postdoctoral fellow in the laboratory of Dr. William J. Lennarz.

Received for publication 9 August 1984, and in revised form 12 November 1984.

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