Analysis of the Membrane-Interacting Domain of the Sea Urchin Sperm Adhesive Protein Bindin†

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ABSTRACT: We have investigated the domain of the bindin polypeptide that selectively associates with gel-phase phospholipid vesicles. We found that small trypsin fragments of bindin retain the ability to selectively associate with gel-phase vesicles. The primary amino acid sequence of bindin suggests that these peptides are derived from the central portion of the polypeptide between residues 77 and 126, which is the most hydrophobic region of bindin. We have also employed 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID) and novel, radioiodinated, photoactivatable derivatives of the polar head group of phosphatidylethanolamine (ASD-PE and ASA-PE) to identify membrane-associated polypeptide segments after the transfer of radiolabel from the probe to the bindin polypeptide. After photolysis, bindin was selectively labeled only from probes incorporated in gel-phase vesicles. The labeling of bindin was much more efficient from the head group probes ASA-PE and ASD-PE (8 and 2% of the total label, respectively) in comparison to the hydrophobic probe TID (less than 0.02% of the total label), suggesting that bindin is localized within the polar part of the bilayer. Protease mapping experiments with V8 protease, trypsin, and endoprotease Lys-C suggest that some of the probe label is distributed along the amino-terminal portion of bindin between residues 1 and 76 and the rest of the label is restricted to the segments between residues 77 and 126 which also selectively bind to gel-phase vesicles. The carboxyl-terminal portion of bindin between residues 127 and 236 is not labeled.

Bindin is an adhesive protein from sea urchin sperm that mediates the species-specific adhesion of sperm to the egg surface during fertilization (Vacquier & Moy, 1977; Glabe & Vacquier, 1977; Glabe & Lennarz, 1979). The binding of sperm is remarkably tenacious; the motile sperm with a 50 μM long flagellum is immobilized by an area of contact that is 0.25 μm in diameter. Bindin specifically binds to sulfated fucose-containing polysaccharides that are found on egg surface proteoglycans (Glabe et al., 1982; DeAngelis & Glabe, 1987). For bindin to serve as a physical bond between the sperm and the egg surface, there must be some mechanism whereby the bindin remains firmly associated with the sperm acrosomal membrane. We have previously discovered that bindin associates selectively with gel-phase phospholipid vesicles in vitro (Glabe, 1985). Bindin associates with both neutral phosphatidycholine and acidic phosphatidylglycerol vesicles, suggesting that the charge of the polar head group is relatively unimportant for the association. The physiological significance of the gel-phase specific association of bindin with phospholipid bilayers is not yet celar. The finding that bindin associates directly with phospholipid bilayers may explain how bindin might associate with the membrane of the sperm acrosomal process during sperm adhesion. Previous studies suggest that gel- and fluid-phase domains exist in plasma membranes (Klausner et al., 1980; Wolf et al., 1981), but this concept remains controversial.

Bindin appears to interact with phospholipid bilayers as a peripheral membrane protein (Glabe, 1985). Bindin readily dissociates from the bilayer as the temperature is raised above the phase transition temperature, and the bindin polypeptide remains accessible to trypsin cleavage after it is associated with the bilayer. The pattern of trypsin cleavage fragments for vesicle-associated bindin is identical with the pattern obtained for bindin in the absence of vesicles. To understand how bindin selectively interacts with membranes, we have attempted to identify the portion of the bindin polypeptide that associates with phospholipid vesicles. Here we report the results of experiments that suggest that proteolytic fragments derived from the central portion of the bindin polypeptide bind preferentially to gel-phase vesicles, and we present complementary evidence which indicates that the amino-terminal and central segments of the bindin polypeptide are in close proximity to the bilayer.

Materials and Methods

Materials. Bindin was isolated as previously described (Vacquier & Moy, 1977). Phosphatidylcholines, dimyristoylphosphatidylethanolamine, trypsin-treated TPCK, and Pronase were purchased from Sigma (St. Louis, MO). V8 protease was from Miles Scientific (Naperville, IL). Phospholipase C and endoprotease Lys-C were both from Boehringer Mannheim (Indianapolis, IN). Iodogen, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP),[1] and the photoactivatable cross-linking reagents sulfosuccinimidyl-2-(*p*-azidosalicylamido)-ethyl-1,3′-dithiopropionate (SASD) and *N*-hydroxysuccinimidyl-4-azidosalicyclic acid (NHS-ASA) were purchased from Pierce (Rockford, IL). 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID), 370 MBq/

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[1] Abbreviations: SASD, sulfosuccinimidyl 3-[[2-(*p*-azidosalicylamido)ethyl]dithio]propionate; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicyclic acid; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine; ASD-PE, *N*-[[[2-(*p*-azidosalicylamido)ethyl]dithio]-propionyl]phosphatidylethanolamine; ASA-PE, *N*-(4-azidosalicyloyl)-phosphatidylethanolamine; PE, phosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; Na-DodSO$_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; PBS, phosphate-buffered saline.

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mmol, was purchased from Amersham (Arlington, IL). Carrier-free $Na^{125}I$ was obtained from ICN Radiochemicals (Irvine, CA). ^{125}I-Labeled protein A was provided by Dr. Daniel J. Knauer. Synthetic peptides were obtained from the peptide synthesis facility, Department of Chemistry, University of California, San Diego.

Isolation of Vesicle-Associated Peptides. Small, unilamellar vesicles of DPPC and DOPC were prepared by sonication of a 5 mg/mL dispersion of lipid in 0.54 M NaCl and 20 mM Tris, pH 8, as described (Glabe, 1985). Bindin was radioiodinated by incubating 400 μg of bindin with 0.25 mCi of $Na^{125}I$ over a 10-μg film of Iodogen at 0 °C for 10 min. The mixture was dialyzed against 0.54 M NaCl, 20 mM Tris, pH 8.0, and 0.02% NaN_3 to remove greater than 90% of the unincorporated label. The radioiodinated bindin (150 μg, 1.2 $\times$ 10^7 cpm) was incubated with either gel- or fluid-phase vesicles (500 μg) for 1 h and then digested with trypsin-treated TPCK at a mass ratio of 1:10 (enzyme to bindin) at room temperature overnight. The vesicle-associated peptides were isolated by flotation on sucrose step gradients. The vesicle solution was adjusted to 50% sucrose by the addition of solid sucrose and transferred to the bottom of a 5-mL ultracentrifuge tube. A step gradient was prepared by overlaying the vesicle solution with 2.0 mL of 30% sucrose in 0.54 M NaCl and 20 mM Tris, pH 8.0, followed by 2.5 mL of 15% sucrose in the same buffer. The samples were centrifuged in a Beckman SW 50.1 Ti rotor at 45 000 RPM for 16 h. After centrifugation, the samples were fractionated by puncturing the tube at the bottom and collecting 0.2-mL fractions. Vesicle-containing fractions were treated with phospholipase C to disrupt the vesicles (Christiansen et al., 1985). Aliquots of the vesicle-associated peptides were analyzed by $NaDodSO_4$ gel electrophoresis as described (Laemmli, 1971), and the dried gels were subjected to autoradiography on Kodak XAR-5 film to visualize radioactive bands. For HPLC analysis, an aliquot of labeled vesicle-associated peptides was mixed with unlabeled tryptic peptides derived from 50 μg of bindin digested under the same conditions described above but in the absence of vesicles. The mixture was dissolved in 0.5 mL of 0.1% trifluoroacetic acid (TFA) in water and injected onto a 0.46 $\times$ 25 cm Vydac C4 reverse-phase column equilibrated with 0.1% TFA water at a flow rate of 0.8 mL/min. Peptides were eluted with a gradient of 0–45% acetonitrile containing 0.1% TFA in 45 min. The elution profile was monitored by UV absorbance at 214 nm, and fractions were collected every minute. For TLC analysis, the fractions were lyophilized, dissolved in 10 μL of water, spotted on cellulose plates (E. Merck) and developed in a 1-butanol/pyridine/acetic acid/water mixture (32.5:25:5:20).

Cross-Linking Experiments. Vesicles used in cross-linking experiments were prepared as described above. In experiments with ASA-PE and ASD-PE the phospholipid mixture also contained 1% phosphatidylethanolamine and was dispersed in phosphate-buffered saline, pH 7.4. For TID labeling experiments, 4 μL of an ethanol solution of TID (4 nmol, approximately 40 μCi) was added to a 100-μL suspension of vesicles (100 μg of phospholipid) in a 1.4-mL microfuge tube and incubated in the dark on ice for 1 h. The vesicle suspension was transferred to a microfuge tube containing 33 μg of bindin and incubated in the dark at room temperature for 1 h and subsequently UV irradiated as described below.

The derivatization of PE vesicles with SASD and NHS-ASA was performed in the dark under a red safe light. The cross-linking reagent was weighed in a tared, foil-wrapped tube and dissolved in dimethylformamide at a concentration of 10 mg/mL. A 2-fold molar excess of cross-linking reagent to the amount of PE was added to a 200-μL aliquot of the vesicles in two separate additions followed by 10-min incubations in the dark. The cross-linker–vesicle suspension was then iodinated as described above for bindin, and the unincorporated label was removed by dialysis. The derivatization of PE was monitored by thin-layer chromatography of the reaction mixture on silica gel F254 plates (E. Merck) in a chloroform/methanol/5 N NH_4OH mixture (65:30:5). The reaction with SASD was apparent as a decrease in the intensity of the fluorescamine-staining spot corresponding to PE concomitant with the appearance of a new spot that incorporated the majority of the ^{125}I label and migrated with a relative mobility of 0.5. SASD incubated in the absence of vesicles gave a spot with a relative mobility of 0.67.

Bindin was added to each vesicle sample at a lipid to protein mass ratio of 3:1, and the samples were incubated in darkness for 1 h at room temperature with shaking. Samples were irradiated with ultraviolet light from a hand-held source (Spectroline Model ENF-260C) from a distance of about 1 cm for 20 min. After photolysis, the radiolabeled, cross-linked bindin was purified by $NaDodSO_4$–polyacrylamide gel electrophoresis. ASA-PE samples were treated with phospholipase C as described above prior to gel purification. Radioactive bands were localized by exposure of the wet gel to X-ray film for 1–2 h and were cut from the gel with a razor blade. The gel slices were lyophilized and digested with trypsin, V8 protease, or endoprotease Lys-C in 100 mM ammonium bicarbonate (pH 8). The slices were incubated at room temperature for 16–18 h. The proteolytic digestion products were eluted twice by the sequential addition of 0.1 mL of water and shaking for 4 h at room temperature. The combined eluates were lyophilized and resolubilized in sample buffer for $NaDodSO_4$–PAGE.

Epitope-Specific Antibodies. Polyclonal antibodies were generated against two synthetic peptides, NH_2-GYPQAMSPQMGGVYNG-COOH and NH_2-GQGYLQG-COOH, corresponding to the amino- and carboxyl-terminal regions of bindin, respectively. The initial immunogen was made by coupling ovalbumin and peptide with SPDP. Briefly, the ovalbumin carrier amino groups were derivatized with SPDP, reduced with excess dithiothreitol, and purified by gel filtration. The peptide α-amino groups were also derivatized with a molar equivalent of SPDP and mixed with the conjugated ovalbumin carrier and allowed to oxidize in air for 4 h. The immunogen was dialyzed against PBS, immulsified in Freund's complete adjuvant, and injected subcutaneously in the backs of female New Zealand white rabbits (10 mg of ovalbumin with 1 mg of peptide/rabbit). After 3 weeks, free peptides (0.5 mg/rabbit) in Freund's incomplete adjuvant were used to boost the rabbits every 2 weeks. Sera from 2 weeks after the second boost (4 mL) was purified by affinity chromatography on a column with immobilized peptide [Reactigel 6× beaded agarose, conjugated according to manufacturer's directions (Pierce)]. After extensive washing with PBS, specific IgG was eluted with 0.2 M glycine, pH 2.5, neutralized with 1 M Tris, pH 8.0, dialyzed against PBS, and used for Western blotting (Towbin et al., 1979). Both epitope-specific IgG and an affinity-purified total bindin antibody were used to probe V8 and trypsin digests carried out as described earlier. ^{125}I-Labeled protein A was used to detect peptides corresponding to the amino and carboxyl termini.

Results and Discussion

Radioiodinated bindin was incubated with either gel-phase DPPC or fluid-phase DOPC vesicles and digested with trypsin

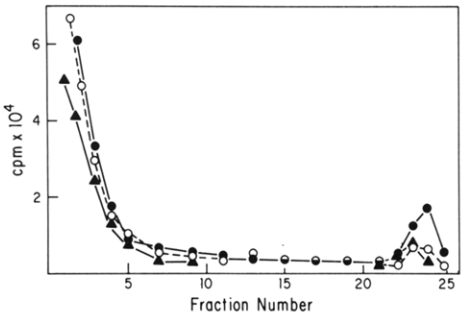

FIGURE 1: Bindin peptides selectively bind to gel-phase phospholipid vesicles after cleavage with trypsin. The sucrose gradient fractionation profile of iodinated bindin peptides in the presence of either gel- or fluid-phase vesicles is shown. (●) Tryptic peptides with gel-phase vesicles; (○) tryptic peptides with fluid-phase vesicles; (▲) pronase peptides with gel-phase vesicles.

Table I: Amino Acid Sequence of *Strongylocentrotus purpuratus* Bindin[a]

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         10          20          30          40          50
●    ●          *           *
YVNTMGYPQA MSPQMGGVNY GQPAQQGYGA QGMGGPVGGG PMGGPPQFGA

         60          70          80          90         100
                                 v       v   v
LPPGQADTDF GSSSSSVDGG DTTISARVMD DIKAVLGATK IDLPVDINDP

                                         |   |
        110         120         130         140         150
●       v   v               v
YDLGLLLRHL RHHSNLLANI GDPAVREQVL SAMQEEEEEE EEDAATGAQQ
                            ^           ^^^^^^ ^^

        160         170         180         190         200
GVLNGNAPGQ AGFGGGGGGG AMMSPQQMGG QPQGMIGQPQ GMGFPHEGMG
                                                      ^

        210         220         230
                            *   *
GPPQGMGMPH QGMGGPPQGM GMPPQGQPYG QGYLQG
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[a] Potential trypsin cleavage sites are indicated by downward arrow heads (v), V8 protease sites are indicated by upward arrow heads, and endoprotease Lys-C sites are indicated by vertical bars. The locations of tyrosine residues are indicated by asterisks.

or Pronase proteases. The vesicle-associated peptides were separated from soluble peptides by flotation on sucrose gradients, as shown in Figure 1. Most of the peptide label does not associate with vesicles and stays at the bottom of the gradient. However, a larger fraction of radioiodinated tryptic fragments of bindin remained associated with the gel-phase DPPC vesicles as compared to fluid-phase DOPC vesicles and migrated to the top of the gradient. Twelve percent of the tryptic peptide radiolabel is associated with DPPC vesicles as compared to 6% of the counts for fluid-phase DOPC vesicles in the experiment shown in Figure 1. A mean value of 18.9 ± 11.5% was obtained for gel-phase vesicles as compared to 5.4 ± 2.6% for fluid-phase vesicles for four independent determinations. The difference between the two populations is significant at the 95% confidence level as analyzed by the *t*-test. In contrast, Pronase-generated oligopeptides and amino acids from radiolabeled bindin do not exhibit any preference for gel- or fluid-phase vesicles. Eight percent of the Pronase-digested ^{125}I-labeled bindin label associates with both DPPC and DOPC vesicles.

The primary amino acid sequence of bindin predicts that complete trypsin digestion should produce two high molecular weight peptides corresponding to the amino- and carboxyl-terminal segments of bindin and five low molecular weight peptides derived from the central portion of the bindin polypeptide between residues 77 and 126 (Table I). Four potential tyrosine iodination sites are located in the amino-terminal

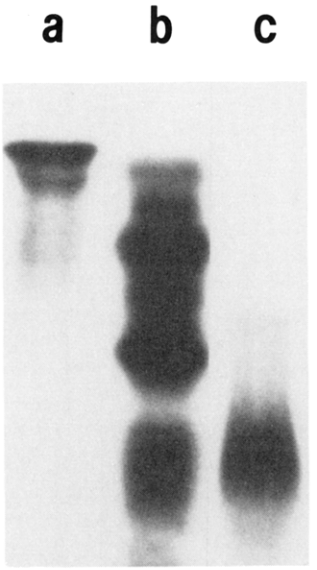

FIGURE 2: Autoradiogram of NaDodSO$_4$–polyacrylamide gel of bindin tryptic peptides. (Lane a) ^{125}I-Labeled bindin prior to digestion; (lane b) total radiolabeled tryptic peptides of bindin prior to fractionation; (lane c) radiolabeled, vesicle-associated bindin peptides after flotation on sucrose gradients.

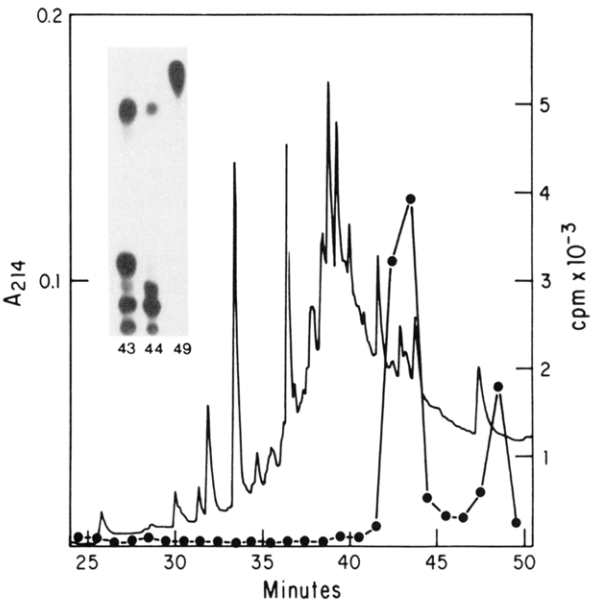

FIGURE 3: Reverse-phase HPLC analysis of radiolabeled, vesicle-associated tryptic peptides. Peptides were fractionated on a gradient from 0 to 45% acetonitrile/0.1% TFA in water over 45 min at a flow rate of 0.8 mL/min. The solid line corresponds to the UV absorbance trace, and the solid circles are the counts associated with each 1-min fraction. (Inset) The radioiodine-containing fractions at 43, 44, and 49 min were dried, and an aliquot was analyzed by TLC.

segment, two in the carboxyl-terminal segment, and one within the central portion of bindin. The vesicle-associated ^{125}I-labeled peptides were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis as shown in Figure 2. Only the low molecular weight fraction of the tryptic peptides remains associated with the gel-phase vesicles after flotation on sucrose gradients. Trypsin does not appear to completely digest bindin, since more than two large radioiodinated bindin peptides are resolved on NaDodSO$_4$–polyacrylamide gels.

To further characterize the vesicle-associated peptides, they were solubilized by phospholipase C digestion of the phospholipid, mixed with peptides labeled with nonradioactive iodine, and fractionated by reverse-phase HPLC as described under Materials and Methods. The majority of the radio-

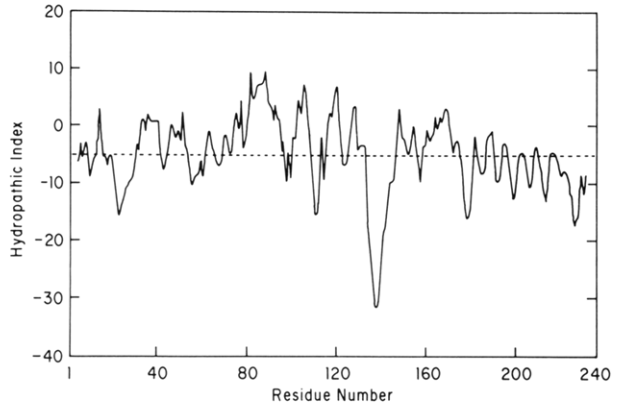

FIGURE 4: Hydropathy plot of bindin. The hydropathic index of the mature bindin sequence was calculated by the algorithm described by Kyte and Doolittle (1982), using a nine amino acid interval. The more hydrophobic regions have a positive score.

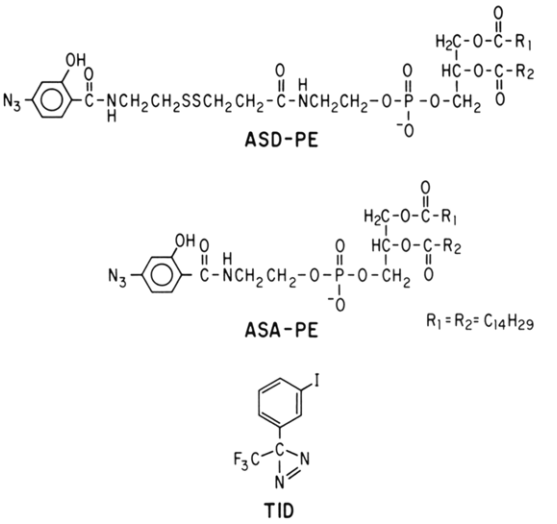

FIGURE 5: Structure of TID and the proposed structures of ASD-PE and ASA-PE.

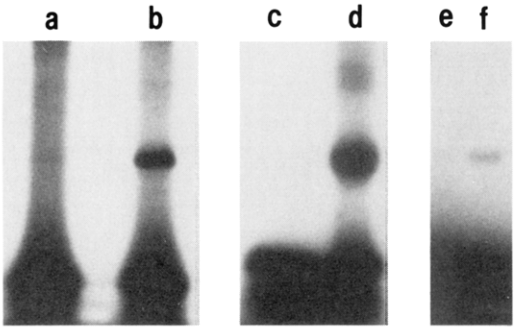

FIGURE 6: Selective labeling of bindin associated with gel-phase vesicles by photoactivatable membrane probes. After photolysis, samples were run on a 12% $NaDodSO_4$–polyacrylamide gel and visualized by autoradiography. (Lane a) Bindin incubated with fluid-phase DOPC vesicles containing radioiodinated ASD-PE; (lane b) bindin incubated with gel-phase DPPC vesicles containing ASD-PE; (lanes c and d) bindin incubated with either gel- or fluid-phase vesicles, respectively, containing radioiodinated ASA-PE; (lanes e and f) bindin incubated with either gel- or fluid-phase vesicles, respectively, containing radioiodinated TID. Bindin is labeled only when it is associated with gel-phase vesicles.

labeled peptides coeluted with a series of four closely spaced peaks detected by UV absorbance at 214 nm (Figure 3). The retention times of the radiolabeled peptides are long in comparison to the bulk of the unlabeled bindin peptides, indicating that they are more hydrophobic. A second peak of radiolabel eluted at the end of the gradient that did not correspond to a UV absorbing peak. Analysis of the radiolabeled fractions by TLC confirms the presence of four distinct radiolabeled components in the major peak (Figure 3, inset). The smaller, late eluting labeled peak migrates at the solvent front on TLC, suggesting that it is a relatively nonpolar molecule. Hydropathy plots of the bindin sequence, calculated as described by Kyte and Doolittle (1982), indicate that the region of bindin between residues 78 and 126 is the most hydrophobic portion of bindin, as shown in Figure 4. Although the size of the vesicle-associated peptides and elution behavior on reverse-phase HPLC are consistent with the interpretation that the low molecular weight peptides are derived from the central portion of the bindin polypeptide, we tried to unambiguously identify the radioiodinated peptides by sequencing. Repeated attempts to sequence this fraction by automated gas-phase Edman degradation failed to yield any sequence information.

The observation that the low molecular weight tryptic peptides bind to vesicles suggests the membrane-binding domain of bindin may reside in the segment between residues 77 and 126. An alternate interpretation we cannot exclude is that proteolysis may produce peptides from an internal hydrophobic region which is not normally exposed at the surface, and these peptides bind artifactually to vesicles. However, the observation that the peptides show a preference for gel-phase vesicles suggests that the binding of the peptides mimics the properties of the intact protein. The experiments described above suffer from a number of limitations. Only radiolabeled peptides are detected, and the fact that a peptide fragment of bindin fails to associate with vesicles does not rule out the participation of this segment in membrane binding by the intact protein.

Because of these limitations, we have utilized an independent strategy to identify the membrane-associated region of bindin using radioiodinated, photoactivatable cross-linking reagents. We used TID (Brunner & Semenza, 1981) and novel photoactivatable derivatives of PE (Figure 5) to label membrane-associated portions of the bindin polypeptide. TID has been used previously to label the hydrophobic domains of proteins that integrate into the hydrocarbon region of the bilayer (Jorgensen & Brunner, 1983) and determine the topology of integral membrane proteins (Page & Rosenbusch, 1988). Bindin was incubated with gel- or fluid-phase vesicles containing [^{125}I]TID and then irradiated with shortwave UV light. After photolysis, the bindin-vesicle samples were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 6). TID specifically labels bindin only from gel-phase vesicles, but the efficiency of labeling is low. Less than 0.02% of the total label is transferred to bindin.

We also prepared radioiodinated, photoactivatable probes for the polar head group region of the bilayer by derivatizing the amino group of PE with ASD and ASA after the incorporation of PE into either gel- or fluid-phase phosphatidylcholine vesicles (Figure 5). The synthetic strategy described under Materials and Methods was based on previous demonstrations of the reactivity of the primary amino group of PE in preformed vesicles with a variety of reagents [reviewed by Heath and Martin (1986)] and was designed to minimize the number of manipulations that must be carried out in the dark. The ASD cross-linker extends approximately 16 Å from the PE amino group and contains a cleavable disulfide bond, whereas the ASA derivative has a shorter, 4-Å "reach" and lacks a disulfide bond.

Bindin was incubated with either gel- or fluid-phase vesicles containing ASD-PE or ASA-PE and photolyzed as described

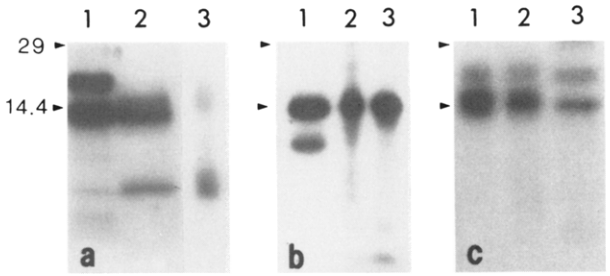

FIGURE 7: Proteolytic mapping of the location of the cross-linked radiolabel. Photolabeled bindin and control radioiodinated bindin samples were digested with either (a) trypsin, (b) V8 protease, or (c) endoprotease Lys-C and run on a 20% $NaDodSO_4$–polyacrylamide gel. (Lane 1) Iodinated bindin; (lane 2) ASD-labeled bindin; (lane 3) ASA-labeled bindin. The arrows indicate the relative migration positions of molecular weight standards: carbonic anhydrase, 29 000; lysozyme, 14 400.

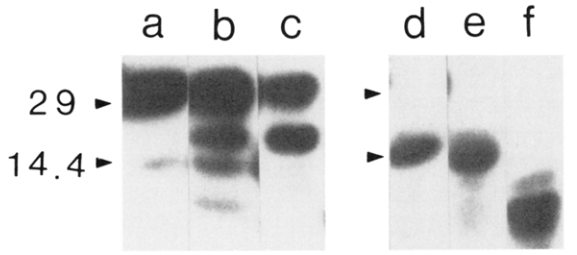

FIGURE 8: Identification of amino- and carboxy-terminal bindin polypeptides by staining with antipeptide antibodies. Bindin samples were cleaved with V8 protease or trypsin, run on 20% $NaDodSO_4$–polyacrylamide gels, and transferred to nitrocellulose. Lanes a–c contained a mixture of bindin and bindin trypsin peptides. Lanes d–f contain bindin V8 peptides. Blots were stained with either antisera against the amino-terminal peptide (lanes a and d), antibindin polyclonal sera (lanes b and e), or antisera against the carboxyl-terminal peptide (lanes c and f). The arrows indicate the relative migration positions of molecular weight standards: carbonic anhydrase, 29 000; lysozyme, 14 400.

above. Only the bindin samples incubated with gel-phase vesicles are selectively radiolabeled by the cross-linking reagents after photolysis (Figure 6), suggesting that the bindin polypeptide is not close enough to the bilayer to react efficiently with the cross-linker when it is incorporated into fluid-phase vesicles. These results also suggest that the ASA-PE and ASD-PE probes are associated with the lipid bilayer and do not diffuse extensively into the aqueous compartment. The efficiency of labeling of bindin by the head-group probes ASA-PE and ASD-PE is much higher than the efficiency of labeling with TID, which is a probe for the hydrophobic core of the membrane. Up to 8% of the probe label is transferred to bindin from ASA-PE, and 2% is transfered from ASD-PE (Figure 6). The differential labeling efficiency is consistent with previous observations which indicate that bindin is peripherally localized and does not integrate extensively within the bilayer. However, comparisons of the labeling efficiency must be made with caution, since the carbenes generated by photolysis of TID have a different reactivity from that of the nitrenes generated from ASA and ASD (Richards & Brunner, 1980).

To determine the location of the label transferred from the cross-linkers, we purified the ASA- and ASD-photolabeled bindin polypeptide from $NaDodSO_4$ gels and digested it with trypsin, V8, and endoprotease Lys-C. The low amount of label transferred to bindin from TID precluded the mapping of its distribution. Figure 7 shows the resulting pattern of labeled fragments after electrophoresis on $NaDodSO_4$–polyacrylamide gels. V8 protease digestion of the ASD-PE and ASD-PE cross-linked products produced a single major band with an apparent molecular weight of 15 000. This fragment corresponds to the amino-terminal fragment of bindin as determined by staining with specific antipeptide antibodies (Figure 8). The photolabeled V8 digestion product also comigrates with the single fluorescent band obtained after V8 digestion of dansylated bindin, confirming that it is the amino-terminal portion of the polypeptide and suggesting that the fragment extends past the lysine residues at residues 83 and 90 (data not shown). The amino terminus and the two lysine residues are the only potential sites of dansylation of the bindin polypeptide. Trypsin digestions of both the ASD-PE- and ASA-PE-labeled bindin produce both a large fragment and low molecular weight labeled fragments. The larger tryptic fragment corresponds to the amino terminus of bindin as determined by specific antibody staining. A larger proportion of the smaller molecular weight fragments is labeled with the shorter reach ASA-PE as compared to ASD-PE. The smaller peptides most likely correspond to fragments between residues 77 and 126, since all of the lysine and arginine residues are located in this region (Table I). The smaller fragments of bindin are stained by the polyclonal antibindin antisera, but not the amino- or carboxyl-terminal-specific antisera (Figure 8). We attempted to sequence the photolabeled digestion products after electrophoretic transfer to activated glass (Aebersold et al., 1986) or poly(vinylidene difluoride) membranes (Matsudaira, 1987). No sequence data were obtained for any of the photolabeled digestion products.

Digestion of the ASD-cross-linked bindin with endoprotease Lys-C, which cleaves on the carboxyl side of lysines, resulted in the production of two large labeled fragments. The same pattern was obtained upon digestion of radiolabeled bindin. Bindin contains two lysine residues separated by six amino acids at positions 83 and 90 (Table I). These results indicate that both the N-terminal and C-terminal endoprotease Lys-C fragments are labeled by the photoactivatable derivative and are consistent with the labeling pattern obtained by trypsin digestion.

We have analyzed the specific interaction of bindin with gel-phase phospholipid vesicles using two independent approaches: isolation from bindin of peptides that selectively associate with gel-phase vesicles and photolabeling of the membrane-associating domain using photoactivatable, radioiodinated lipid probes. Both approaches indicate that the membrane-associating domain of the bindin is localized within the central portion of the polypeptide. We have isolated small trypsin fragments of bindin that retain the ability to selectively associate with gel-phase phospholipid bilayers. These fragments are most likely derived from the central portion of the bindin polypeptide, since all of the six potential trypsin sites are contained in the region from residue 77 to residue 126. Protease mapping of the location of the cross-link radiolabel confirms these results. The region extending between residues 77 and 126 is labeled by the phospholipid head group cross-linking probes as is the region from residue 1 to residue 77. The carboxyl-terminal portion of bindin from residue 127 to residue 236 is not labeled, suggesting that this part of the bindin polypeptide does not closely approach the bilayer. From the cross-linking data, it is not yet clear how extensive the membrane-associating domain of bindin is or whether this domain is a continuous stretch of the bindin polypeptide. The area of membrane contact may include the entire polypeptide from residue 1 to residue 127 or it may be relatively small, centered across the two lysine residues at positions 83 and 90. Since proteolytic fragments of bindin retain the ability to selectively bind to gel-phase vesicles, perhaps these remaining questions can be answered by use of synthetic peptide ana-

logues of bindin or truncated recombinant bindin polypeptides.

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Aggregation-Related Conformational Change of the Membrane-Associated Coat Protein of Bacteriophage M13

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ABSTRACT: The state of the coat protein of bacteriophage M13, reconstituted into amphiphilic media, has been investigated. The in situ conformation of the coat protein has been determined by using circular dichroism. Minimum numbers for the protein aggregation in the system have been determined after disruption of the lipid–protein system and subsequent uptake of the protein in cholate micelles. The aggregational state and conformation of the protein were affected by (1) the method of coat protein isolation (phenol extraction vs cholate isolation), (2) the nature of amphiphiles used (variation in phospholipid headgroups and acyl chains), and (3) the ratio of amphiphiles and protein. Under all conditions, phenol-extracted coat protein was in a predominantly β-structure and in a highly aggregated polymeric form. Cholate-isolated coat protein was initially oligomeric and contained a substantial amount of α-helix. Below an aggregation number of 20, this protein showed a reversible aggregation with no change in conformation. Upon further aggregation, a conformational change was observed, and aggregation was irreversible, resulting in predominantly β-structured coat protein polymers. This effect was observed upon uptake in phospholipids at low lipid to protein molar ratios (L/P ratios) and with phosphatidylcholines (PC) and phosphatidic acids (PA) containing saturated acyl chains. After reconstitution in phospholipds with unsaturated acyl chains and with phosphatidylglycerols (PG) at high L/P ratios, the original α-helix-containing state of the coat protein was maintained. Cross-linking experiments demonstrated that the β-polymers are able to form reversible superaggregates within the vesicle system. An aggregation-related conformational change mechanism for the coat protein in phospholipid systems is proposed.

M13 bacteriophage is an *Escherichia coli* specific filamentous phage. The rod-shaped virion is composed of a cylindrical protein capsid, which mainly consists of the gene 8 product: the major coat protein [see reviews by Ray (1977), Makowski (1984), and Rashed and Oberer (1986)]. During infection, the viral DNA is released into the cytoplasm of *E. coli*, while its coat protein is inserted into the cytoplasmic membrane (Pratt et al., 1969). It is generally assumed that, in vivo, parental as well as newly synthesized coat protein is stored oriented as an integral (trans)membrane protein (Smilowitz, 1974; Wickner, 1975; Ohkawa & Webster, 1981). During the combined assembly–extrusion process, the major coat protein is assembled around the viral DNA.

The primary structure of the coat protein [molecular weight (MW)[1] 5240] is given in Figure 1. Three specific domains can be distinguished: an acidic N-terminus (residues 1–20)

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; PG, phosphatidylglycerol; DMPA, dimyristoylphosphatidic acid; DOPA, dioleoylphosphatidic acid; PA, phosphatidic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; L/P ratio, lipid to protein molar ratio; CD, circular dichroism; MW, molecular weight; DMA, dimethyl adipimidate; DMS, dimethyl suberimidate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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