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Characterization of the membrane-associating domain of the sperm adhesive protein, bindin

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Bindin is an adhesive protein that mediates the binding of sea urchin sperm to the egg during fertilization. Bindin selectively associates with gel-phase phospholipid vesicles in a peripheral fashion. Bindin interacts specifically with sulfated fucan on the egg's surface, and directly with the phospholipid bilayer of the sperm. Analysis of a series of deletion mutants of recombinant bindin was undertaken to define the membrane associating domain of bindin. Recombinant and sperm bindin display nearly identical binding kinetics to gel-phase phospholipids and have equivalent saturation points of approx. 250 lipid molecules per molecule of bindin. Deletion mutants of bindin which contain residues 75–130 retained specific membrane binding activity. Synthetic peptides corresponding to residues 69–130, and 92–130 also display gel-phase specific membrane association. This region is highly conserved within four different species of bindin molecules. Circular dichroism spectroscopy of synthetic peptides corresponding to residues 92–130 and 69–130 suggests that a distinct change in conformation takes place upon binding liposomes. Taken together, these data indicate that the membrane binding activity of bindin resides within this highly conserved region of the bindin molecule.

Introduction

Bindin, an adhesive protein present in the acrosomal granule, is released during the acrosome reaction and is believed to mediate species-specific adhesion of sperm to the egg vitelline envelope [1,2]. The adhesion of sperm is so tenacious that the 50 μm beating flagellum is immobilized by an area of bindin in contact with the egg that is 0.25 μm in diameter. In order to maintain this adhesion, bindin also must interact strongly with the membrane of the acrosomal process of the sperm. Bindin selectively binds to gel-phase phospholipid vesicles in vitro in a peripheral fashion [3]. The structure of the phospholipid headgroup has relatively little effect on the ability of bindin to associate with vesicles. The interaction of bindin with phospholipid vesicles is not calcium dependent.

Other evidence suggests that bindin interacts with membranes as a peripheral membrane protein. Bindin binds to dimyristoylphosphatidylcholine vesicles below the phase transition temperature (t_m) of 23°C, but dissociates from the vesicles if the temperature is raised above the t_m [3]. Crosslinking reagents attached to the external polar headgroups of liposomes efficiently label bindin, while crosslinking reagents which partition in the hydrophobic region of the bilayer do not significantly react with bindin [4]. This evidence, in conjunction with data which shows that bindin remains sensitive to proteinase digestion after it is bound to phospholipid vesicles strongly support this suggestion [3]. Proteolytic mapping of the sites of labeling by photoactivatible lipid derivatives of membrane associated bindin suggest that the N-terminal and central two-thirds of bindin are involved in membrane binding [4]. Here we report the results of experiments which suggest that the lipid-binding domain of sperm bindin is contained entirely between amino acids 75 and 130, and that a synthetic peptide of the region 92–130 displays gel phase specificity and lipid binding ability.

Materials and Methods

Materials

Bindin was isolated from *Strongylocentrotus purpuratus* sperm as previously described [2]. Phosphatidylcholines and trypsin-TPCK were purchased from Sigma

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(St. Louis, MO). Carrier free $Na^{125}I$ was obtained from ICN Radiochemicals (Irvine, CA), and Amersham (Arlington, IL). Synthetic peptides were synthesized with a continuous flow semi-automatic instrument and Fmoc chemistry as previously described [5]. Peptides were purified by reverse phase HPLC on a Vydac C4 column using a gradient of water and acetonitrile with TFA (0.1%) and characterized by matrix-assisted laser desorption mass spectroscopy. Small, unilamellar vesicles of DPPC and DOPC were prepared by sonication of a 5 mg/ml dispersion of lipid in 0.15 M NaCl, 20 mM Tris-HCl (pH 8) as described [3]. Transmission electron microscopy of a negatively stained sample showed that greater than 95% of the vesicles were unilammellar.

Preparation of recombinant bacterial bindin

Recombinant bindin was produced as a fusion protein with the lambda cII gene product as previously described [6]. The cII portion of the resultant recombinant protein is cleaved by the bacterial omp-T proteinase to produce final products of mature recombinant bindin containing an extra one or four N-terminal amino acids derived from the probindin processing site [6,7]. In order to purify recombinant bindin, the induced bacterial cells were washed with 33 mM Tris-HCl (pH 8.0), and then the outer membrane was disrupted by treating the bacterial cells with 0.15 mM EDTA and 0.02 mg/ml lysozyme in an isotonic (22%) sucrose solution for 20 min. The spheroplasts were sedimented at $3000 \times g$ for 10 min, and the supernatant was discarded. The pellet was then frozen and thawed two times to lyse the spheroplasts, and sonicated in 20-s bursts with a Heat Systems Ultrasonics, sonicator at a power setting of 3.5 (on ice) for approx. 3 min in a small volume of 6.7 M urea, 20 mM Tris-HCl (pH 8.0), 50 mM NaCl (1 ml per 100 ml original bacterial culture). The protein mixture was then diluted to 0.4 M urea and fractionated by ammonium sulfate precipitation. The recombinant bindin precipitates between 10% and 20% ammonium sulfate (from saturated). Recombinant bindin was further purified by reverse phase HPLC on a Vydac C-4 column. The bindin-containing pellet was redissolved in 4 M urea, 1% trifluoroacetic acid (TFA) and injected onto a 0.46×25 cm Vydac C4 reverse phase column equilibrated with 8% acetonitrile/0.1% TFA and 92% water/0.1% TFA at a flow rate of 1.0 ml/min. The bindin was eluted with a gradient of 8%–100% acetonitrile containing 0.1% TFA over 45 min. The elution profile was monitored by UV absorbance at 215 nm and fractions were collected every minute. The bindin containing fractions were lyophilized and resuspended in 0.15 M NaCl and 20 mM Tris-HCl (pH 8.0). Protein concentrations were determined using the method of Lowry et al. [8].

Preparation of amino and carboxyl terminal deletion mutants of recombinant bindin

Amino terminal deletions were prepared by digesting the pBindin plasmid with the restriction enzyme *Hpa*I, whose site is 18 codons from the start of the mature bindin sequence [6,9]. The linearized plasmid was digested with Bal 31 for different time intervals, and the ends were subsequently repaired with T4 polymerase. The resulting products were digested with *Bgl*II, and the different sized fragments were then ligated into the bindin expression vector. Because the deletions occurred at the N-terminus, we screened the colonies with anti-bindin antibodies to select clones in the correct reading frame. Over 400 amino terminal clones were screened with anti-bindin antibody, and approximately one third of these were antibody positive. Carboxyl terminal deletions were prepared by cutting the pBindin plasmid at the *Bgl*II site located at the termination codon of the bindin open reading frame, and digesting with Bal 31 as described above. The vector was ligated together with a linker containing stop codons in three reading frames. 56 clones were selected which contained the linker, and the majority of these expressed recombinant bindin of reduced length. The deletion mutants were expressed in *Escherichia coli* and purified by the method described above for recombinant bindin.

Binding assays

Bindin, recombinant bindin, and bindin analogs were radioiodonated by incubating 20 μg of protein with 0.20 mCi of $Na^{125}I$ over a 1 μg film of Iodogen at 0° for 10 min. Proteins and peptides 61 amino acids or more in length were dialyzed against 0.15 M NaCl, 20 mM Tris-HCl (pH 8.0) to remove greater than 90% of the unincorporated label. Peptides and trypsin fragments smaller than 61 amino acids were purified on a C18 SEP-PAC cartridge (Waters Associates, Milford, MA). The labeling mixture was diluted 5-fold in water containing 0.1% TFA and applied to cartridges preequlibrated in this solvent. The free iodine was removed by washing the cartridges with 30 ml of water/0.1% TFA, and the peptide was eluted with 50:50 ACN/water containing 0.1% TFA. The peptide was lyophilized to dryness, and resuspended in 0.15 M NaCl, 20 mM Tris-HCl (pH 8). Binding measurements were made in triplicate and performed by incubating 0.5 μg of labeled protein with 25 μg or 100 μg of liposomes and 80 μg of ovalbumin, in a total volume of 40 μl 0.15 M NaCl, 20 mM Tris-HCl (pH 8). To prepare binding isotherms, the labeled bindin was diluted with unlabeled bindin in the incubation mixture. Vesicle-associated proteins and peptides were isolated by flotation on sucrose step gradients. 155 μl of 70% (w/v) sucrose was added to the vesicles-peptide solutions to adjust the concentration to 55% sucrose, and

transferred to the bottom of a Beckman 5 × 41 mm ultra-clear Ultracentrifuge tube. A step gradient was prepared by overlaying this solution with 300 μl of 30% (w/v) sucrose in 0.15 M NaCl, 20 mM Tris-HCl (pH 8) followed by 150 μl of 15% (w/v) sucrose in the same buffer. The samples were centrifuged in a Beckman SW 50.1 Ti rotor at 100000 × *g* for at least 15 h. The vesicles, and vesicle associated peptide float to the top of the gradient and were removed by pipetting off the top 350 μl of the centrifuge tube (equal to 1/2 the total volume of the tube). The top 1/3 of the centrifuge tube was clipped off with scissors and washed with 1% SDS to remove any vesicles adsorbed to the tube, and to evenly disperse the lipids in the solution. The counts present in the top 350 μl were considered vesicle bound, and the counts present in the bottom half of the tube were considered unbound. Because in many cases there was some residual free iodine present, and because in some cases the labeled bindin protein was not entirely homogeneous, volume equivalent samples from the top and bottom of centrifuge tubes were run on $NaDodSO_4$ gels [10] and the dried gels were subjected to autoradiography on Kodak XAR-5 film to visualize radioactive bands. The radioactive bands corresponding to bindin or bindin analogs were then cut out of the gels, and counted individually to calculate the amount of bound and free bindin protein. Binding data was analyzed statistically using Student's *t*-test. For the 38 and 28 amino acid bindin peptides, a Tricine gel system [11] was utilized for better resolution of the peptide from free iodine.

Circular dichroism of sperm bindin and related peptides

Circular dichroism spectra of sperm bindin and the 69–130 and 92–130 bindin synthetic peptides were obtained using a Jasco 700 instrument. The CD data were expressed in terms of mean residue ellipticity, and the spectra presented are the average of at least 10 scans per sample. Spectra were obtained between the range of 195–250 nm. A sample of bindin isolated from sperm, $1.3 \cdot 10^{-3}$ M, was analyzed as well as the 69–130 bindin peptide ($7.4 \cdot 10^{-3}$ M), and the 92–130 bindin peptide ($6.0 \cdot 10^{-6}$ M). Both peptides were also analyzed in the presence of 5 mg/ml DPPC between the same wavelengths. Due to a significant increase in light scattering of the liposomes in the presence of sperm bindin, equivalent data was not obtained for the native protein. In all cases, the buffer used was 0.15 M NaCl, 20 mM Tris-HCl (pH 8).

Results

Interaction of bindin with phospholipid vesicles

The stoichiometry of the bindin-liposome interaction was determined for both recombinant bindin and bindin isolated from sperm (Fig. 1). Both sperm and

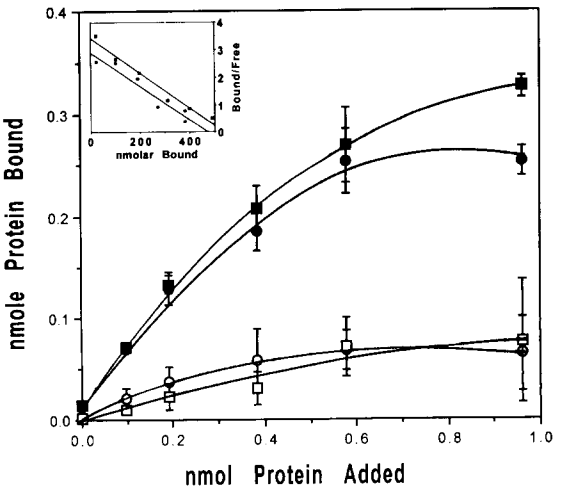

Fig. 1. Binding of sperm bindin and recombinant bindin to phospholipid vesicles. Purified sperm bindin (●) or recombinant bindin (■) was incubated with a constant amount of gel-phase DPPC phospholipid vesicles. Each data point was measured in triplicate and the data are presented as the mean and the standard deviation for each point. Sperm bindin and recombinant bindin both show an maximum molar ratio of 250 lipid molecules to 1 bindin molecule. At higher concentrations, a slight, but reproducible decrease in the amount of binding is observed consistently for sperm bindin in these assays. Sperm bindin (○) and recombinant bindin (□) bind fluid phase DOPC liposomes to a significantly lesser extent. The inset figure represents a Scatchard presentation of the data, and shows that both sperm and recombinant bindin have a binding constant of $1.6 \cdot 10^{-7}$.

recombinant bindin display a distinct preference for gel-phase vesicles as previously described for sperm bindin [3]. The amount of vesicle-associated bindin increases with increasing amounts of bindin added until a maximum is reached. At low concentrations of bindin (< 0.6 nmol) there is no statistically significant difference in the binding of sperm and recombinant bindin ($P > 0.18$). At the highest concentration examined, there is a small, but statistically significant difference between sperm and recombinant bindin ($P < 0.007$). Although the explanation for this difference is not yet clear, this may be due to bindin self aggregation and the fact that sperm bindin is less soluble than recombinant bindin (unpublished observation). These data indicate that the binding constant for both sperm bindin and recombinant bindin to DPPC vesicles is $1.6 \cdot 10^{-7}$ with R^2 values of 0.92 and 0.97, respectively. At the maximum level of binding, approx. 1 nmol of protein is bound per 250 nmol lipid. Similar binding maximums and stoichiometries were obtained for sperm and recombinant bindin. If only the external leaflet of the bilayer is accessible to bindin, these results would suggest that each bindin polypeptide covers an area occupied by 125 lipid molecules, which is equivalent to approx. 5000 Å^2. If bindin is a globular protein, its radius would be approx. 40 Å and the cross-sectional area it would occupy is also approx. 5000 Å^2. This would imply that bindin's association with the phospho-

lipid vesicle, at maximal binding, is as a monolayer of protein with the entire surface of the vesicle covered by bindin molecules. The addition of bindin to the phospholipid vesicles does cause a slow leakage of small molecules, such as terbium and dipicolinic acid across the bilayer (unpublished data). In the event that both the inner and outer leaflets of the liposome are accessible to bindin, then at maximal binding the vesicle surface would not be completely covered with bindin.

Binding properties of recombinant bindin deletion analogs and synthetic peptides

Having determined that sperm and recombinant bindin demonstrate similar vesicle binding properties, we prepared carboxyl and amino terminal deletion mutants from the recombinant bindin expression vector. Clones expressing different sized bindin deletion analogs were selected and purified (Fig. 2). The clones were sequenced to determine the extent of the deletions. The largest deletion obtained for the amino terminus was δ18–75 and carboxyl terminus was δ119–236. The purified bindin deletion analogs were tested for membrane binding activity as described for bindin above. The binding activity was determined by a competition assay where increasing amounts of unlabeled bindin or recombinant bindin analogs were added to compete for protein binding on the liposome surface.

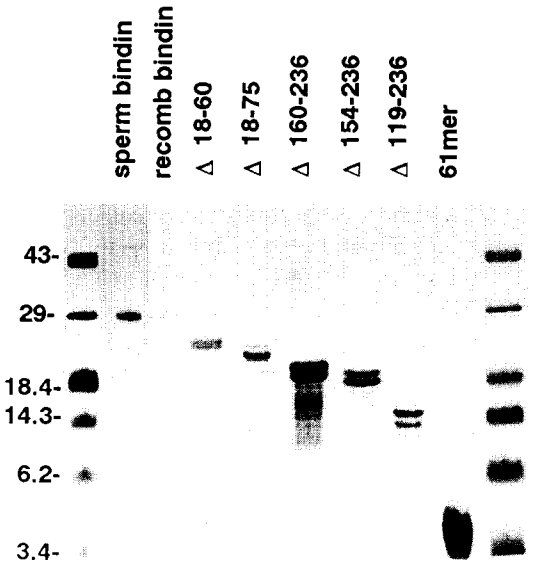

Fig. 2. SDS-PAGE of reverse-phase HPLC purified bindin and bindin deletion analogs. Lane 1, molecular weight markers; Lane 2, sperm bindin; Lane 3, recombinant bindin; Lanes 4 and 5, amino terminal deletion analogs; Lanes 6–8, carboxyl terminal deletion analogs; Lane 9, 61 residue synthetic peptide corresponding to residues 69–130 of *S. purpuratus* bindin. These purified fractions were labeled with ^{125}I and used for binding experiments. There are two bands apparent for recombinant bindin and recombinant bindin deletion analogs because the proteinase that cleaves the mature product from the fusion proteinain recognizes two cleavage sites, three amino acids apart.

The results of these experiments are shown in Fig. 3. All of the amino terminal deletion products yielded a competition isotherm comparable to sperm and recombinant bindin, and demonstrated selectivity for gel phase vesicles. The larger carboxyl terminal deletion analogs (δ154–236, δ160–236) demonstrated the same binding activity and selectivity while the smallest deletion analog (δ119–236) was specific for gel phase liposomes, but showed reduced binding activity in comparison to the full length protein. These results indicate that residues 1–75 and 154–236 can be deleted without a significant loss of vesicle binding activity or gel phase selectivity. This implies that the phospholipid binding activity of bindin resides within residues 75–154 of the native molecule. We prepared several clones that contained larger deletions of the bindin coding sequence and clones designed to express residues 75–119, but found that they could not be expressed at a high enough level to facilitate purification and analysis of their biochemical properties.

As an alternative approach to expressing residues 75–120 as a recombinant fusion protein, we prepared synthetic peptides and trypsin fragments of bindin. A synthetic peptide spanning residues 69–130 retained binding activity comparable to full-length sperm bindin (Fig. 3). The other synthetic peptides and trypsin fragments were purified, labeled with ^{125}I, and a small amount was incubated with excess gel and fluid phase liposomes (Fig. 4). Peptides spanning residues 92–130, and 92–120 displayed reduced binding activities. Additionally, the peptide spanning amino acids 92–120 did not demonstrate significant selectivity for gel phase vesicles. As a control, the large C-terminal fragment of bindin produced by trypsin digestion corresponding to residues 126–236 did not bind to lipid vesicles of either type as previously suggested [4]. An internal bindin trypsin fragment corresponding to residues 91–108 also did not display significant binding to either type of liposome. This data suggests that a minimum requirement for preferential gel phase vesicle binding includes amino acids 92–130 of the mature bindin sequence. Together with the results from the deletion analogs, these results support the conclusion that the region between residues 69–130 is required for *both* binding activity *and* gel phase specificity (Fig. 5).

Circular dichroism of synthetic peptides

To explore the possibility that membrane association may stabilize a particular secondary structure of the peptides, circular dichroism measurements were made before and after vesicle association (Fig. 6). The spectra of the 92–130 and 69–130 peptides in the absence of vesicles showed a local absorbance minima at 202.5 nm. The spectrum of bindin also displayed a local minima of smaller magnitude in this region, suggesting that native bindin may contain a similar sec-

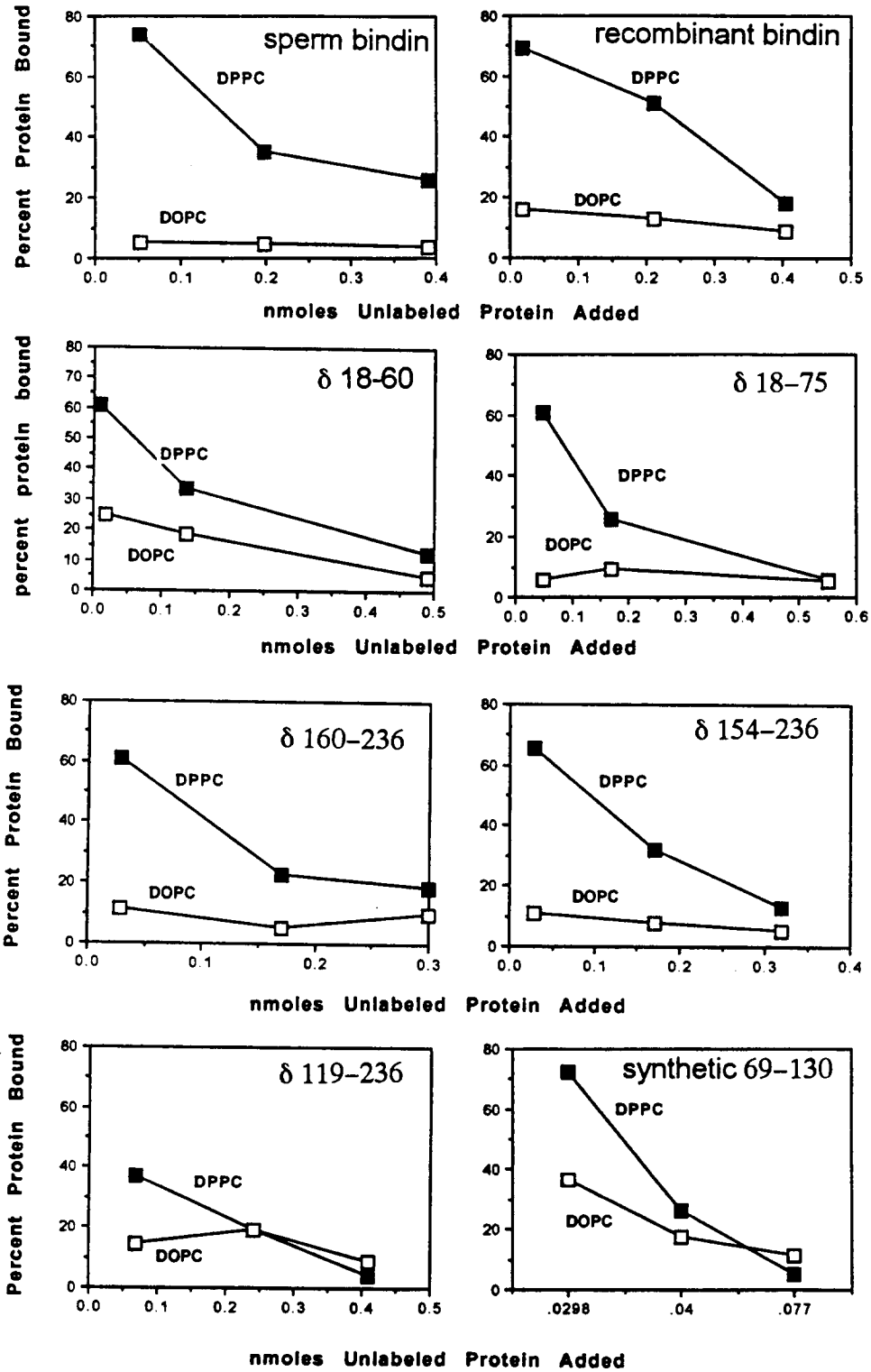

Fig. 3. Binding of bindin, recombinant deletion analogs of bindin and a synthetic peptide corresponding to residues 69–130 to phospholipid vesicles. Sperm bindin, recombinant bindin and deletion analogs of bindin were labeled with ^{125}I, and incubated with gel and fluid phase phospholipid vesicles. Increasing amounts of unlabeled bindin or corresponding deletion of recombinant bindin were added to completely compete for the phospholipid vesicle surface. The only deletion analog demonstrating significant deviation from the trend and reduction in binding capabilities was δ 119–236. The synthetic peptide spanning the region 69–130 displays an affinity for phospholipid vesicles similar to that of recombinant bindin. While it displays a significant affinity for fluid phase vesicles, it does differentiate between gel and fluid phase vesicles.

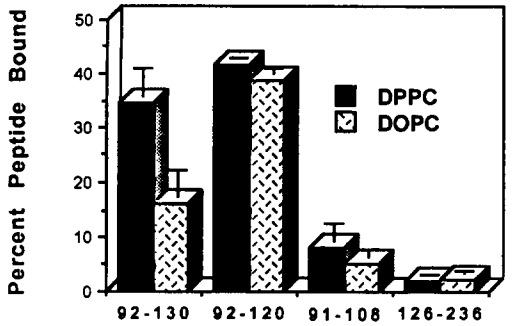

Fig. 4. Binding of bindin peptides to phospholipid vesicles. Synthetic peptides of bindin were labeled with ^{125}I and incubated with gel and fluid phase phospolipid vesicles. This experiment was done under conditions where the phospholipid vesicles were present in vast molar excess to the labeled protein. The synthetic peptide spanning the region 92–130 demonstrates reduced binding affinity, but still displays a distinct preference for gel phase liposomes. The synthetic peptide spanning the region 92–120 binds equally well to gel phase and fluid phase liposomes. An internal trypsin fragment of bindin (90–108) shows a greater than 5-fold reduction in binding ability. The C-terminal trypsin fragment of bindin (126–236) does not contain a significant part of the conserved region of sperm bindin and is unable to bind liposomes to any significant extent.

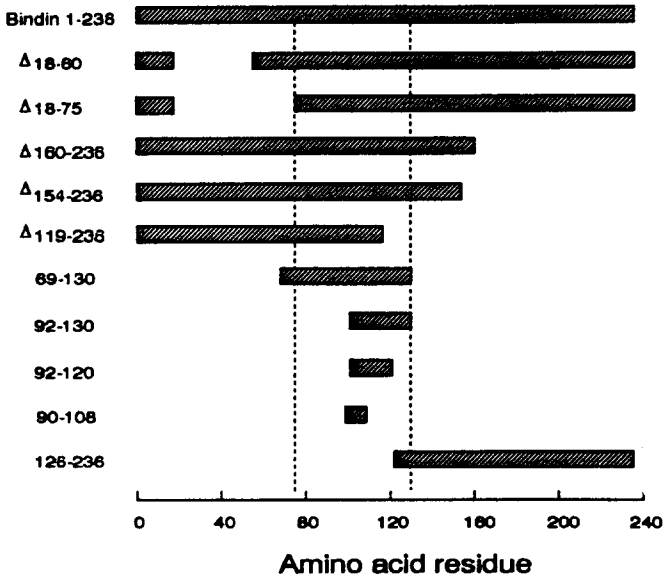

Fig. 5. Summary of the structures of recombinant bindin deletion analogs, synthetic peptides and trypsin fragments used in these studies. The bracketed area contains the region of sperm bindin that is highly conserved in four species of urchin.

ondary structure, but because of the longer chain length of sperm bindin, the secondary structure demonstrated by one domain may be averaged out over the whole protein length. We were also interested in determining whether the binding of the peptide is associated with a change in secondary structure. Both peptides showed a significant shift in the local minima from 202.5 nm to 206 nm upon vesicle binding, and in the case of the 92–130 peptide, the shift in wavelength was accompanied by an increase in magnitude as well. The change

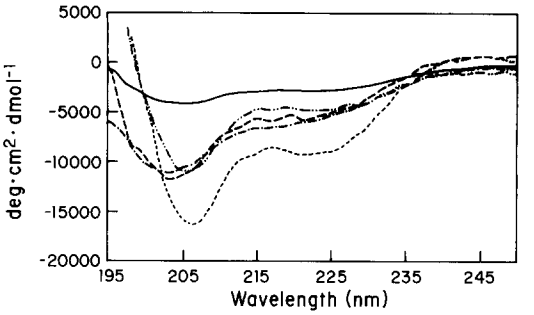

Fig. 6. Circular dichroic spectra of sperm bindin and bindin peptides. Circular dichroic spectra of sperm bindin (———), 69–130 synthetic peptide (– – –) and 92–130 synthetic peptide (–·–·–) were obtained in 0.15 M NaCl, 20 mM Tris-HCl (pH 8.0). A minimum of 10 measurements were made for each wavelength and the average was used to obtain the final curve. Circular dichroic spectra of the 69–130 (–··–) and 92–130 (------) synthetic peptides were also made in the same buffer in the presence of an excess of DPPC liposomes. The deep trough at 204 nm is shifted to 207 nm, and for the 92–130 peptide, is increased in magnitude.

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                    10        20        30
S.p. YVNTMG         YPQAMSPQMGGVNY GQPAQQGYGAQGMGG
S.f. YGNQGN         YPQAMNPQSRGVNY GQPAQQGYGAQGMGGAFGGGQGMGGAVRGGQGMGG
L.v. YGNMN          YPQPMNQPMGGGNYPGQPPQQNYAPQGMGG
A.p. YAQGAGGMQGGYGYPQA      GGAQYGGQPVQ GYMNQGPPMG

          40        50                                  60        70
S.p. PVGGGPMGGPPQFGAL       PPGQADTD                    FGSSSSSVDGGDTTISARV
S.f. AVGGG        QPGAF     SPGEAEADNA                  DYDEYSDSLDEGDTTISAAV
L.v. PVGGGGMGGAVGAGAMGGPVGGGGGGMGGPVGGANGIGESVEDEMSVDSDYSSLGGE  TTISAKV
A.p.  QRPAAAGPAGGFGAPQGQPPVGQPIGEAAGGGEFLGEPGVGGE SEFAEYSSSIGEGE TINAEV

      80        90        100       110       120       130       140
S.p. MDDIKAVLGATKIDLPVDINDPYDLGLLLRHLRHHSNLLANIGDPAVREQVLSAMQEEEEEEEEDA
S.f. MDDIKAVLGATKIDLPVDINDPYDLGLLLRHLRHHSNLLANIGDPAVREQVLSAMQEEEEEEEEDA
L.v. IQDIKNLLGATKIDLPVDINDPYYLGLLLRHLRHHSNLLANIGDPEVREQVLSAMQEEEEEEENDA
A.p. MEKIKAVLGATKIDLPVDINDPYDLGLLLRHLRHHSNLLANIGDPEVRNQVLTAMQEEEEEEEQDA

          150       160                                 170       180
S.p. ATGAQQGVLNG   NAPGQAGFGG                          GGGGGAMMSPQQMGGQPQGMIG
S.f. ANGVRQNVLNNINANAPGNAGYGG QGGMGAFGGGGGGMGAIGGGGGAMMGQQGMGGVPQRMGG
L.v. ANGVRENVLNNL  NAPGQGGYGGTQGGMRGGAGG
A.p. ANGVRDNVLNNLN EGPGA                             GAVAGAAMAAGMPPYPRAQGG

               190       200       210       220       230
S.p.        QPQGMGFPHEGMGGPPQGMGMPHQGMGGPPQGMGMPPQGQPYGQGYL QG
S.f.        QPQG                                    NAYN  QGYR QG
L.v.          GMMGN QGMGGQG                           YN  QGYM QG
A.p.  GMRVGGQPQN      PMGG                           NAYNPMTGYRQQG
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Fig. 7. Amino acid sequence of mature bindins from four species of sea urchins. The abbreviations are as follows: S.p., *Strongylocentrotus purpuratus* bindin [9]; S.f., *Strongylocentrotus franciscanus* bindin; L.v., *Lytechinus variagatus* bindin [15]; A.p., *Arbacia punctulata* bindin [16]. The region between residues 75 and 130 is highly conserved in all four species.

in the secondary structure of the synthetic peptides upon contact with gel phase liposomes is consistent with the idea that the lipid binding region of sperm bindin is contained between residues 75–130, and suggests that membrane binding may also result in a change of the conformation of bindin as well. Peptides which do not bind to vesicles do not demonstrate a change in CD spectra. A 20 residue peptide from the 'tat' gene product of HIV and the β-amyloid 1–28 peptide both demonstrated CD spectra that were superimposable with their spectra in the presence of gel phase liposomes (data not shown).

Discussion

Previous investigations suggested that the lipid binding domain of sperm bindin is located in the amino-terminal two thirds of the mature bindin polypeptide [4]. Crosslinking experiments with radioiodinated, photoactivatible derivatives of the phospholipid head group indicate that the amino terminal two thirds of bindin is accessible to labeling. The goal of these studies was to define the region of interaction with greater precision and to determine the stoichiometry of bindin-vesicle association. Bindin is in an insoluble, particulate form as it exists within the acrosomal vesicle of the sperm. We expected to find that phospholipid vesicles were coated with a bilayer or multilayer of bindin at maximal saturation, due to a bindin–lipid interaction at the liposome surface, and a bindin–bindin interaction between layers of bindin. The binding stoichiomentry indicates that approx. 125 lipid molecules on the surface of the liposome interact with one bindin molecule, which suggests that bindin coats the vesicle as a monolayer. This suggests the possibility that the bindin–lipid interaction may be stronger than the bindin–bindin interaction, and that the bindin redistributes from the bindin particle to coat the vesicle surface. The apparent dissociation constant for both sperm and recombinant bindin is $1.6 \cdot 10^{-7}$. The same approximate lipid to protein ratio has been obtained for the binding of apolipoprotein E to phosphatidylcholine vesicles which has an apparent dissociation constant of $5.9 \cdot 10^{-7}$ [12].

Analysis of the binding properties of truncated bindin analogs and synthetic peptides suggest that the mature bindin region between amino acids 75–130 contains the lipid binding domain. Sperm bindin and recombinant bindin display the same binding isotherms with both fluid and gel phase liposomes, suggesting that the recombinant bindin is not denatured. Analysis of the binding properties of deletion mutants has shown that residues 1–74 and 131–236 can be deleted without loss of membrane binding activity and gel-phase specificity. A synthetic peptide spanning the region 69–130 displays binding activity comparable to bindin and a preference for gel phase liposomes. Together, these results indicate that the membrane-binding domain is contained within the region 75–130 of the mature bindin sequence, which is consistent with the interpretation of the crosslinking studies.

One of the carboxyl-terminal deletion analogs, δ119–236, displays a 30% decrease in binding as compared to intact sperm bindin and recombinant bindin, although it still retains the ability to display a gel phase preference. Synthetic peptides of the region 92–130 and 92–120 also have a reduced binding capacity of about 40%. The synthetic peptide spanning to 130 demonstrates a gel phase preference, while the peptide that is truncated at 120 does not display a significant preference for gel phase versus fluid phase liposomes. The loss of gel phase specificity suggests that residues 120–130 may contribute to the gel-phase selectivity of binding. However, we cannot rule out the possibility that removal of these residues results in improper folding of the peptide and therefore loss of gel-phase selectivity.

The region between amino acids 75–130 in the *S. purpuratus* bindin sequence is highly conserved within four species of sea urchins (Fig. 6). This suggests that bindin isolated from these sea urchins may also bind lipids in a highly similar, if not identical manner. Because the region between residues 75–130 appears to constitute the membrane binding domain of bindin, we would like to obtain more information about the structural basis for this interaction. Circular dichroic spectra have shown that this region in synthetic peptides shares common characteristics with the native protein. Circular dichroic spectra of synthetic peptides corresponding to residues 69–130 and 92–130 display a small, broad trough at approx. 220 nm and a marked, deeper trough around 206 nm which is also observed in bindin. The spectrum of the 92–130 peptide changes upon addition of gel-phase vesicles, suggesting that it may undergo a conformation change. The trough at 206 nm shifts to 208 nm and its intensity increases. Although the precise structural interpretation of the CD spectra is not yet clear, the spectra of sperm bindin and the synthetic bindin peptides is remarkably similar to that reported for proteolytic fragments of human lipoprotein B-100 [13] and apolipoprotein E [12] in association with vesicles. These proteins also binds liposomes in a peripheral manner, and human lipoprotein B-100 may contain several lipid binding regions which appear to be widely distributed throughout the molecule. The apolipoprotein family, which binds lipid vesicles in a peripheral manner, is believed to assume an amphipathic α-helical conformation where close orientation of the zwitterionic polar headgroup of the liposome interacts with the ion pairs of the protein and non-polar side chains associate with the hydrophobic core of the bilayer. [14]. Orientation of residues 75–130 of the bindin protein into a helical wheel format shows that if this region of bindin has alpha-helical character, it too would form an amphipathic helix, allowing for the same type of interaction between bindin and the liposome surface. Although bindin may also interact with the surface of the bilayer as an amphipathic α-helix, this remains to be experimentally verified.

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