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Identification of the Protein 4.1 Binding Site to Phosphatidylserine Vesicles†

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ABSTRACT: Previous studies have shown that protein 4.1 is a multifunctional protein that binds to spectrin, actin, glycophorins, the anion channel protein, and phosphatidylserine (PS). In this report, we have characterized the binding of protein 4.1 and its major proteolytic fragments to phospholipid vesicles. Pure ^{125}I-labeled protein 4.1 was incubated with PS liposomes, and the free protein 4.1 was separated by ultracentrifugation. Protein 4.1 bound to PS liposomes with a high affinity. At saturation, there was 9×10^{-3} pmol of protein 4.1 bound/pmol of PS with a K_d of 3.3×10^{-7} M. When the protein 4.1 containing liposomes were examined in an electron microscope, the protein 4.1 was found uniformly decorating the vesicles in a rosettelike fashion. Among peripheral membrane proteins tested (spectrin, actin, ankyrin, and protein 4.1), protein 4.1 showed the highest level of binding to PS. The binding of protein 4.1 to PS, one of the principal phospholipids of the inner half of the lipid bilayer, was considerably higher than the binding to phosphatidylcholine, that is principally located in the outer half of the lipid bilayer. To identify the structural domain of protein 4.1 involved in binding to the phospholipids, a mixture of proteolytic fragments of protein 4.1 was incubated with PS liposomes. The liposomes selectively retained the 30-kilodalton (kDa) basic domain of the protein, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/isoelectric focusing. The 30-kDa fragment was purified from chymotryptic digests of protein 4.1 by ion-exchange chromatography on DEAE 52. The purified 30-kDa peptide of protein 4.1 competitively inhibited the binding of ^{125}I-labeled protein 4.1 to PS. These data indicate that protein 4.1 is capable of forming high-affinity associations with PS. These associations may be important in the maintenance of normal red cell structure and function.

The red blood cell membrane skeleton contains four major proteins: spectrin, actin, protein 4.1, and protein 4.9 (Cohen, 1983). Protein 4.1 is composed of two very similar polypeptide chains of 80 and 78 kilodaltons (kDa) which have essentially identical peptide maps (Goodman et al., 1982). The larger polypeptide differs from the smaller by having an extension at its carboxyl terminus (Leto & Marchesi, 1984). The structure of protein 4.1 was recently studied by limited proteolysis employing chymotrypsin (Leto & Marchesi, 1984). Protein 4.1 was cleaved primarily in three central locations which generated intermediate-size peptides that were found to represent specific structural and functional domains of the original molecule. The protein 4.1 molecule displays unusual polarity, and its 56- and 46-kDa chymotryptic digests contain the 30-kDa basic peptide that appears to be resistant to further digestion by chymotrypsin (Leto & Marchesi, 1984). Protein

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4.1 stabilizes the spectrin–actin association (Ungewickell et al., 1979; Cohen & Foley, 1982) and binds to the cytoplasmic segment of band 3 (Pasternak et al., 1985) and glycophorins (Mueller & Morrison, 1981; Anderson & Lovrien, 1984). Protein 4.1 binding to glycophorin A is enhanced by a specific phospholipid, phosphatidylinositol 4,5-bisphosphate (Anderson & Marchesi, 1985). Phosphatidylserine, a major constituent of the inner lipid bilayer, was recently shown to interact with protein 4.1 (Sato & Ohnishi, 1983).

In this paper, we compared the binding of protein 4.1 to the skeletal proteins spectrin, actin, and ankyrin. We provide direct ultrastructural and biochemical evidence that protein 4.1 binds with a high affinity to phosphatidylserine (PS) vesicles. We further identify the basic 30-kDa fragment as a site of protein 4.1 binding to PS.

Experimental Procedures

Materials. ^{125}I-Labeled Bolton–Hunter reagent and [^{3}H]triolein were purchased from New England Nuclear. Bovine brain phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) were purchased from Avanti and were 99% pure by thin-layer chromatography (TLC). Chymotrypsin was purchased from Worthington Co. All other chemicals were reagent grade or better.

Isolation and Labeling of Protein 4.1. Human erythrocyte protein 4.1 was purified by high-salt extaction (Tyler et al., 1980; Cohen & Foley, 1984). Ghosts were prepared from 1 unit of fresh blood by hypotonic lysis according to the method of Dodge et al. (1963). Ghosts in 3 mM phosphate buffer (pH 8.0) were treated with 0.5 mM diisopropyl fluorophosphate (DFP) at 0 °C for 10 min to inactivate endogenous proteases. Ghosts were introduced to 0.1 mM phosphate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM dithiothreitol (DTT), pH 8.0, for 30 min at 37 °C to remove spectrin. Vesicles were resuspended in 150 mM NaCl, 5 mM phosphate, 0.1 mM EDTA, and 0.1 mM DTT (pH 7.6) to remove band 6 and pelleted by centrifugation at 20000*g* for 15 min. The vesicles were treated with 1 M NaCl, 5 mM phosphate, 0.1 mM EDTA, 2 mM DFP, and 0.1 mM DTT (pH 8.0) at 37 °C for 30 min to extract ankyrin and protein 4.1. These vesicles were removed by centrifugation at 200000*g* for 35 min, and the supernatant was dialyzed against 20 mM NaCl, 5 mM phosphate, 0.1 mM EDTA, and 0.1 mM DTT (pH 7.6). Protein 4.1 was purified by ion-exchange chromatography on Whatman DE 52 phosphate (pH 7.6). The purity of protein 4.1 was tested by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Protein 4.1 was radiolabeled with ^{125}I-labeled Bolton–Hunter reagent and dialyzed extensively against 150 mM NaCl, 5 mM phosphate, 0.1 mM EDTA, 0.1 mM DTT, and 0.15 mM sodium azide.

Limited Digestion of Protein 4.1. In order to identify the binding domain of protein 4.1 to PS vesicles, we employed limited digestion. Purified protein 4.1 was partially digested by α-chymotrypsin (1:25–1:500 enzyme to substrate ratio), in 150 mM NaCl, 5 mM phosphate (pH 6.5), 0.1 mM EDTA, 0.1 mM DTT, and 0.15 mM sodium azide at 0 °C for 30 min, as described (Leto & Marchesi, 1984). The digestion was terminated by 1 mM DFP.

Preparation of Phospholipid Vesicles. Large unilamellar phospholipid vesicles were prepared by the reverse-phase evaporation technique (Szoka & Papahadjopoulos, 1978) as modified (Wilschut et al., 1980) for quantitation and isolation of protein–phospholipid complexes. Vesicles formed by this technique have a high aqueous space to lipid ratio and encapsulate a high percentage of the initial aqueous phase. The phospholipids (10 mg) were dissolved in 0.7 mL of diethyl ether (prepared by distillation), to which 0.3 mL of 150 mM NaCl, 5 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) (pH 7.1), 1 mM DTT, 1 mM EDTA, and 0.15 mM sodium azide was added. The nontransferable phospholipid [^{3}H]triolein was sometimes added in trace amounts (0.007%) to allow quantitation of vesicles in the protein 4.1–phospholipid binding assay. The preparation was sonicated for 5 min in a bath-type sonicator (lab supply, T-80-80-IRS) under an argon atmosphere. The mixture was then placed on a rotary evaporator, and the diethyl ether was removed under reduced pressure. After the vesicles were made, they were uniformly sized through polycarbonate filters (Olson et al., 1979). Phospholipid vesicles prepared by these methods were stored under argon at 0 °C for up to 7 days to prevent aggregation and oxidation.

Purified bovine brain PS liposomes used for electron microscopic studies were sonicated (Heat System Ultrasonics, Model 185F, 200 W) for 30 min at 0 °C under a nitrogen atmosphere to prevent oxidation. Under these conditions, small unilamellar vesicles are formed (Szoka & Papahajopoulos, 1980). Metal debris and undispersed lipid were removed by centrifugation at 200000*g* for 30 min at 4 °C. The phospholipid concentration in the various liposome preparations was determined by phosphate analysis by the method of Ames (1962). The phospholipid vesicle preparations were tested for oxidation derivatives by measurement of malondialdehyde (Gutteridge, 1977).

Protein 4.1–Phospholipid Vesicle Binding Assay. Specifically, purified ^{125}I-labeled protein 4.1 (0.01–0.20 mg/dL) was incubated with 6.2 nmol of PS or PC liposomes in a total volume of 0.1 mL of 5 mM TES, pH 7.1, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT for 30 min at 0 °C in siliconized plastic tubes. To separate the liposome-bound ^{125}I-labeled protein 4.1 from the unbound protein, a stock solution of 40% Ficoll-400 was added to the incubation mixture to a final concentration of 25% Ficoll. In 0.8-mL silicon-coated ultracentrifuge plastic tubes, 0.1 mL of the 25% Ficoll/incubation mixture was layered carefully. On the incubation mixture, 0.6 mL of 20% Ficoll-400 was layered, and the gradient was centrifuged in an SW 50.1 rotor at 200000*g* for 35 min at 4 °C (Rybicki et al., 1984). The tubes were frozen in liquid nitrogen and cut with a razor blade 1.0 cm from the interface. Protein 4.1 binding to liposomes was determined by the radioactivity of ^{125}I-labeled 4.1 and the [^{3}H]triolein-labeled liposome content of the 20% Ficoll layer, to where the protein–lipid complexes floated.

Isoelectric Focusing/SDS–Polyacrylamide Gel Electrophoresis of Chymotryptic Digests of the 4.1 Protein. To determine which domain of the protein 4.1 molecule associates with PS vesicles, the protein 4.1 chymotryptic digests bound to PS were analyzed by isoelectric focusing/SDS–polyacrylamide gel electrophoresis (IEF/SDS–PAGE). Protein 4.1 was incubated for 30 min at 0 °C with α-chymotrypsin, in a 1:100 enzyme to substrate ratio, in 5 mM TES (pH 6.5), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT. The digestion was terminated by addition of 2 mM DFP. Protein 4.1 digests (0.2 mg/mL) were incubated with 1.2 mM PS vesicles in 5 mM TES (pH 6.5), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT at 0 °C for 30 min. Liposome-bound protein 4.1 peptides were separated from unbound peptides by equilibrium density centrifugation on a discontinuous Ficoll-400 gradient. The PS–digest mixture was mixed with 40% Ficol-400 to a final concentration of 25% (w/v), in a total volume of 1 mL. The PS–protein 4.1 digest was layered into siliconized, 13 × 51 mm polyallomer ultracentrifuge tubes followed by a layer of

3.5 mL of 20% Ficoll-400. The gradient was centrifuged for 35 min at 200000*g*. The PS-bound peptide complexes were aspirated from the top, and the unbound digests were collected from the bottom of the tube. The digested samples bound to PS were mixed with 2.5% SDS and 1 M DTT and preheated to 100 °C for 10 s to dissociate the PS from the fragments. Then the digests were dialyzed against 9.5 M urea, 2% NP-40, and 5% β-mercaptoethanol for 16 h at 22 °C. The samples were electrofocused for 16 h at 400 V in 4% polyacrylamide tube gels (0.4 × 10 cm) containing 0.8% pH 4–6, 0.8% pH 5–7, and 0.4% pH 3–10 carrier ampholytes (Bio-Rad) as described by O'Farrell (1977). SDS gel electrophoresis in the second dimension was performed on 10% acrylamide gels.

Isolation of a Basic 30-kDa Fragment from Protein 4.1 Digests. The basic 30-kDa fragment of protein 4.1 was isolated to assess the role of this fragment in the binding of intact 4.1 to PS vesicles. Purified protein 4.1 was digested by α-chymotrypsin at a 1:50 enzyme to substrate ratio in 5 mM TES (pH 6.5), 20 mM NaCl, 1 mM EDTA, and 1 mM DTT at 0 °C for 30 min. The digestion was terminated by 2 mM DFP. To isolate the basic 30-kDa fragment of protein 4.1, the digests in 5 mM TES (pH 6.5), 20 mM NaCl, 1 mM EDTA, and 1 mM DTT were subjected to ion-exchange chromatography on 0.7 mL of DEAE 52. Adsorbed digests were eluted with a 10-mL linear gradient of 40–200 mM NaCl.

Inhibition of Protein 4.1 Binding to Phosphatidylserine by a 30-kDa Fragment of Protein 4.1. Phosphatidylserine vesicles at a final concentration of 0.62 nmol were incubated with 0.025–0.20 mg/dL purified 30-kDa digest of protein 4.1 and 0.0005–0.075 mg/dL ^{125}I-labeled protein 4.1 in 5 mM TES (pH 6.5), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT for 30 min at 0 °C. To separate the ^{125}I-labeled protein 4.1 bound to PS from the unbound protein 4.1, 40% Ficoll was added to a final concentration of 25% Ficoll. In 0.8-mL silicon-coated ultracentrifuge plastic tubes, 0.1 mL of the 25% Ficoll/incubation mixture was layered carefully. On the incubation mixture, 0.6 mL of 20% Ficoll-400 was layered, and the gradient was centrifuged in an SW 50.1 rotor at 200000*g* for 35 min at 4 °C. The tubes were frozen in liquid nitrogen and cut with a razor blade 1.0 cm from the interface. The radioactivity of the ^{125}I-labeled protein 4.1 bound to PS and the unbound radioactivity were counted (in duplicates) in the upper and lower fractions, respectively.

Ultrastructural Identification of Protein 4.1–PS Complexes by Low-Angle Rotary Shadowing. Protein 4.1 (0.2 mg/dL) was incubated with small PS vesicles (0.5 mM) in 5 mM TES (pH 7.1), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT at 0 °C for 30 min under an argon atmosphere. Aliquots were added directly to glycerol at 0 °C to yield a glycerol concentration of 60%. Samples of protein 4.1 or protein 4.1–PS complexes were sprayed onto freshly cleaved mica, dried under vacuum, and shadowed on a rotating stage with platinum–carbon from an electron bombardment gun (Balzers BAF 400D) at a shadow angle of 6° as described (Tyler & Branton, 1980). Replicas were coated with support film of carbon, floated onto distilled water, mounted on 400-mesh copper grids, and examined with a JEOL JEM 100S electron microscope at 60 kV. Samples of pure PS vesicles in 60% glycerol were sprayed onto freshly cleaved mica which was previously prepared by immersion into boiling 0.1% Alcian blue 8 GX, for 2 min, rinsed with distilled water, and air-dried, to promote lipid vesicle adhesion to the mica (Granger et al., 1982). The sprayed PS vesicles were dried and shadowed in the same way as pure protein 4.1 (Tyler & Branton, 1980). Pure PS vesicle replicas were floated off the mica and mounted on 400-mesh copper grids and studied in the same way as pure protein 4.1 and protein 4.1–PS complexes as described (Cohen et al., 1986).

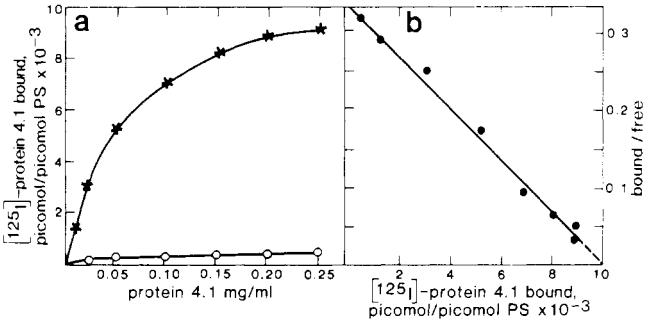

FIGURE 1: Binding of protein 4.1 to phosphatidylserine vesicles. (a) Purified ^{125}I-labeled protein 4.1 was incubated with 6.2 nmol of phosphatidylserine (asterisks) or phosphatidylcholine (O) liposomes in 5 mM TES (pH 7.1), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT for 30 min at 0 °C. Protein 4.1 bound radioactivity to phospholipid vesicles was determined as described under Experimental Procedures. (b) The data of protein 4.1 binding to phosphatidylserine vesicles are plotted according to the Scatchard equation, $B/F = nK - BK$, where B is picomoles of protein 4.1 bound for picomoles of phosphatidylserine, F is the concentration of unbound protein 4.1 in nanomolar, n is the total number of binding sites, and K is the equilibrium constant.

RESULTS

Interaction of Protein 4.1 with Phosphatidylserine and Phosphatidylcholine Vesicles. Quantitation of protein–lipid binding employed equilibrium density centrifugation of protein–unilamellar lipid vesicle mixtures on a discontinuous Ficoll-400 gradient. In this system, radiolabeled protein 4.1 interacting with vesicles floats to the top of the 20% Ficoll gradient, while unbound protein 4.1 remains in the 25% Ficoll gradient. This method resulted in more than 95% recovery of the protein–lipid complexes and less than 1% diffusion of the labeled protein to the top of the gradient.

Protein 4.1 binding to PS or PC vesicles reached equilibrium at less than 10 min of incubation, at 0 or 37 °C. The results of protein 4.1 binding to PS and PC vesicles, which are dominant constituents of the inner and outer lipid bilayers, respectively, are shown in Figure 1. The binding of protein 4.1 to PS vesicles at isotonic conditions, pH 7.1, 0 °C, was remarkably higher in comparison to PC vesicles. At a protein 4.1 concentration of 0.1 mg/mL, 7×10^{-3} pmol of protein 4.1/pmol of PS was bound while in the case of PC, 0.4×10^{-3} pmol of protein 4.1/pmol of PC was bound. These results were comparable to the results obtained with unlabeled protein 4.1 and dextran-loaded vesicles which were sedimented and quantitated by the method of Lowry (Lowry et al., 1951) (unpublished data). The protein 4.1–PS association was evaluated for nonspecific binding by acid and heat denaturation of protein 4.1. The binding following preincubation of protein 4.1 with 10 mM acetic acid was 8.5% of the binding of untreated protein 4.1. On the other hand, preheating of radiolabeled protein 4.1 to 90 °C for 10 min did not decrease the binding to a significant degree.

The binding of protein 4.1 to PS seemed saturable; however, at protein 4.1 concentrations of more than 0.3 mg/mL, there was a marked decrease of the liposomal complexes recovery. The decrease in the protein 4.1–PS complex recovery was probably due either to a leak of the Ficoll-400 into the vesicles or to formation of 4.1 protein aggregates on the vesicle surface. From the binding isotherm of protein 4.1 to PS, at saturation there was 9×10^{-3} pmol of protein 4.1 bound/pmol of PS as shown in Figure 1a. Scatchard analysis of the protein 4.1–PS

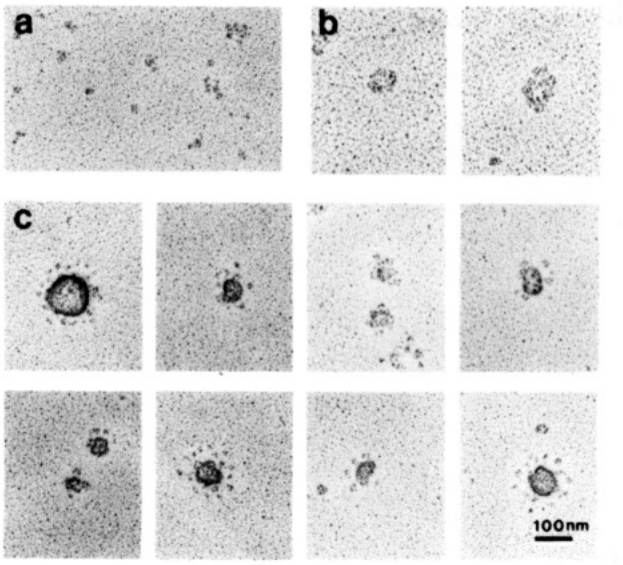

FIGURE 2: Electron micrographs of low-angle rotary-shadowed platinum–carbon replicas of protein 4.1 and phosphatidylserine vesicles. Protein 4.1 (0.2 mg/dL) was incubated with PS vesicles (0.5 mM) in 5 mM TES (pH 7.1), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT at 0 °C for 30 min. The sample was diluted in glycerol and sprayed and shadowed as described under Experimental Procedures, Bar = 100 nm. (a) Protein 4.1. (b) Phosphatidylserine vesicles. (c) Protein 4.1–phosphatidylserine complexes.

isotherm data yielded a linear relationship from which a dissociation constant (K_d) of 3.3×10^{-7} M was calculated.

Phosphatidylserine Interaction with Spectrin, Actin, and Ankyrin. To evaluate the relative affinity of PS to selected skeletal proteins, PS vesicles were incubated in isotonic conditions at 0 °C for 180 min, under an argon atmosphere. Protein 4.1 showed the highest binding to PS vesicles in comparison to spectrin dimers, ankyrin, actin, and albumin. At a protein 4.1 concentration of 0.1 mg/mL, 7×10^{-3} pmol of protein 4.1 was bound/pmol of PS. The binding of spectrin dimers was considerably lower; at the same mass concentration, 3×10^{-5} pmol of spectrin dimers was bound/pmol of PS. Ankyrin, actin, and human albumin did not show any significant binding under these experimental conditions.

Ultrastructure of Protein 4.1–Phosphatidylserine Complexes. Ultrastructural evaluation of protein 4.1–PS complexes was done to demonstrate that this association was genuine and not an artifact. As shown in Figure 2a,b, the diameter of the protein 4.1 molecule (50–70 Å) and its globular form are consistent with the published values (Tyler et al., 1979). The diameter of the PS liposomes (less than 500 Å) was consistent with measurements of the liposomes by negative staining (data not shown) and with published values for the sonicated small unilamellar vesicles (Szoka & Papahadjopoulos, 1980). Protein 4.1 was found to associate with the surface of PS liposomes (Figure 2c).

Identification of a 30-kDa Fragment of Protein 4.1 as a Binding Site to Phosphatidylserine Vesicles. To identify the domain of protein 4.1 that binds to PS vesicles, we employed intermediate-sized peptides of protein 4.1 produced by limited proteolysis by α-chymotrypsin, using an enzyme to substrate ratio of 1:500. Separation of these chymotryptic peptides by two-dimensional IEF/SDS–PAGE resolved them into basic and acidic components (Figure 3a). The 56-, 46-, and 30-kDa basic peptide family and the 24-, 34-, and 50-kDa acidic

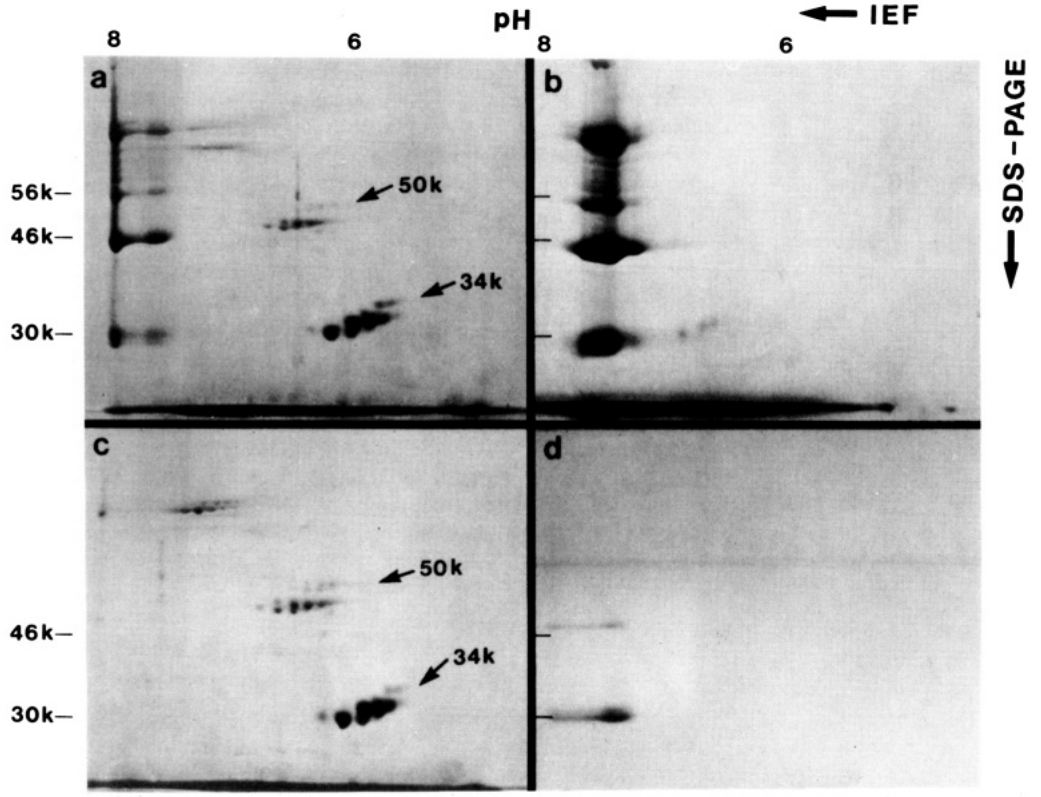

FIGURE 3: Two-dimensional electrophoretic analysis of peptides generated by restricted proteolysis of protein 4.1 by α-chymotrypsin. The digest was produced from 500 μg of protein 4.1 incubated with 1.0 μg of α-chymotrypsin, at 0 °C, for 30 min. Protein 4.1 digests (0.2 mg/mL) were incubated with 1.2 mM PS vesicles in 5 mM TES (pH 6.5), 120 mM NaCl, 1 mM EDTA, and 1 mM DTT at 0 °C for 30 min. Liposome-bound protein 4.1 peptides were separated from unbound peptides by equilibrium density centrifugation on a discontinuous Ficoll-400 gradient. (a) The whole protein 4.1 digest [1:500 (w/w) enzyme to substrate ratio]. (b) Protein 4.1 digest peptides bound to phosphatidylserine vesicles consist of the basic fragments (56, 46, and 30 kDa). (c) The protein 4.1 fragments unbound to phosphatidylserine vesicles are mainly the groups of peptides of 50 and 34 kDa. (d) Protein 4.1 digested at 1:100 (w/w) enzyme to substrate ratio was incubated with phosphatidylserine vesicles. The predominant peptide bound to the vesicles is the basic 30-kDa fragment.

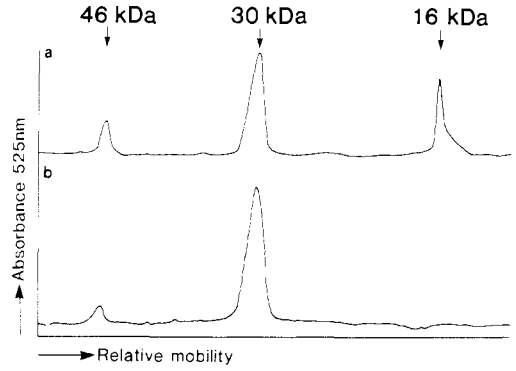

FIGURE 4: Electrophoretic analysis of protein 4.1 digests purified by ion-exchange chromatography. Protein 4.1 was digested by α-chymotrypsin at a 1:50 (w/w) enzyme to substrate ratio in 5 mM TES (pH 6.5), 20 mM NaCl, 1 mM EDTA, and 1 mM DTT at 0 °C for 30 min. The digestion was terminated with 2 mM DFP, and the digests were loaded on a DEAE 52 column. The digests were eluted by a linear gradient of 40–200 mM NaCl. The fractions were analyzed by 12% polyacrylamide–SDS gel electrophoresis followed by densitometric scanning. (a) Densitometric scan of protein 4.1 digest before DEAE column chromatography. The peak of 30-kDa fragments represents 59% of total digests. (b) The fraction containing purified 30-kDa fragments after DEAE 52 column chromatography. The peak of 30-kDa fragments represents 92% of the total proteins in the fraction.

peptide family were similar to previously published digests (Leto & Marchesi, 1984).

The chymotryptic digests of protein 4.1 were incubated with PS vesicles, and the peptides bound to PS were separated by equilibrium density centrifugation on a discontinuous Ficoll-400 gradient. The PS-bound digests which were collected from the top of the gradient and the unbound fragments, which remained at the bottom, were evaluated by two-dimensional IEF/SDS–PAGE. As shown in Figure 3b, the PS-bound peptides included exclusively the 56-, 43-, and 30-kDa basic peptide group, while the unbound peptides, shown in Figure 3c, included mainly the acidic group of peptides. The basic region of the protein 4.1 molecule gives rise to a group of peptides that are relatively less susceptible to proteolytic degradation than the acidic peptides (Leto & Marchesi, 1984). The basic 56- and 46-kDa peptides of protein 4.1 include the 30-kDa peptide in their structure (Leto & Marchesi, 1984). To test whether the 30-kDa basic peptide is capable of binding PS vesicles, we obtained a 30-kDa peptide-enriched mixture by chymotryptic digestion of protein 4.1 at a higher enzyme to substrate ratio (1:100). As shown in Figure 3d, the 30-kDa basic peptide indeed bound PS vesicles.

Inhibition of Protein 4.1 Binding to Phosphatidylserine by the Basic 30-kDa Fragment. Further enrichment of the 30-kDa basic peptide was essential for inhibition studies in order to establish the specificity of the 30-kDa fragment for the binding of protein 4.1 to PS. A 92% enriched mixture of the 30-kDa basic fragment was obtained by ion-exchange chromatography as shown in Figure 4. As shown in Figure 5a, the purified basic 30-kDa fragment inhibited the binding of the ^{125}I-labeled protein 4.1 to PS vesicles by 90% at 106-fold molar excess of the 30-kDa fragment. This inhibition effect was specific because preincubation of the PS vesicles with albumin did not affect the ^{125}I-labeled protein 4.1–PS association. The inhibition of protein 4.1 association with PS vesicles was dependent on the basic 30-kDa concentration as reflected in the Dixon plot (Dixon, 1953) shown in Figure 5b.

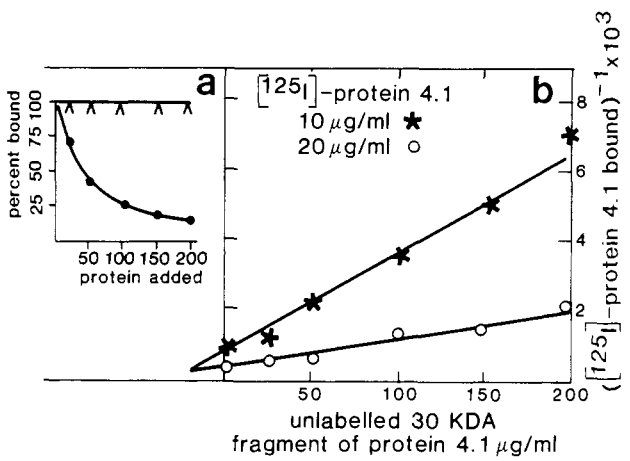

FIGURE 5: Competitive inhibition of protein 4.1 binding to phosphatidylserine vesicles by the basic 30-kDa fragment of protein 4.1. (a) Phosphatidylserine vesicles (3.1 nmol) were preincubated for 30 min with increasing amounts of albumin (asterisks) or with the basic 30-kDa fragment of protein 4.1 derived from α-chymotrypsin digestion of protein 4.1 (●). ^{125}I-Labeled protein 4.1 was added and the incubation continued for an additional 30 min in 5 mM TES (pH 6.5), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT at 0 °C. Ninety percent inhibition of the binding of ^{125}I-labeled protein 4.1 to phosphatidylserine is achieved with a 106-fold molar excess (200 μg/mL) of the basic 30-kDa fragment of protein 4.1 (100% = 1.2 pmol of protein 4.1 bound/pmol of phosphatidylserine). (b) Dixon plot (Dixon, 1953) shows competitive inhibition of ^{125}I-labeled protein 4.1 to phosphatidylserine when PS vesicles (3.1 nmol) were incubated with increasing amounts of purified unlabeled basic 30-kDa fragment of protein 4.1, prior to the addition of 10 μg/mL (asterisks) or 20 μg/mL (○) ^{125}I-labeled protein 4.1.

Discussion

The results presented in this paper provide both direct biochemical and ultrastructural evidence that protein 4.1 interacts with PS and confirm previous reports (Sato & Ohnishi, 1983; Rybicki et al., 1984). The protein 4.1–PS association is of relatively high affinity ($K_d = 3 \times 10^{-7}$ M). We further show that this association is mediated by the basic 30-kDa peptide which is derived from the N-terminal end of protein 4.1 as evidenced by the ability of the 30-kDa fragment to compete with the native 4.1 molecule in binding PS. The affinity of protein 4.1 to PS is considerably higher than spectrin, actin, or ankyrin. In addition, binding of protein 4.1 to PS is 15 times higher than PC. We further show that acid treatment resulted in marked decrease in the binding of protein 4.1 to PS. In contrast, heat denaturation did not alter the 4.1 binding, presumably due to the relative stability of the 30-kDa basic fragment involved in the 4.1–lipid interaction.

The significance of the protein 4.1–PS interaction in the maintenance of the structural integrity of the lipid bilayer remains to be established. Recently, oxidation of protein 4.1 was shown to be associated with a 4-fold decrease in PS binding (Rybicki & Schwartz, 1985) and thus possibly destabilize the lipid bilayer. Furthermore, protein 4.1 deficient red cells were found to have an altered pattern of membrane lipid distribution, with significant enrichment in the outer lipid leaflet content of PS (Schwartz et al., 1985). Whether or not the altered protein 4.1–lipid interaction plays a role in development of elliptocytosis and hemolysis that is found in subjects deficient in this protein (Alloisio et al., 1985) is unknown. It is tempting to speculate that protein 4.1, by virtue of its high binding capacity for PS in the erythrocyte membrane, contributes to the maintenance of the lipid asymmetry in the red cell membrane.

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Interaction of Sperm Histone Variants and Linker DNA during Spermiogenesis in the Sea Urchin†

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ABSTRACT: Several physical properties of sea urchin spermatid chromatin, which contains phosphorylated Sp H1 and Sp H2B histone variants, and mature sperm chromatin, in which these histones are dephosphorylated, were compared. Density, thermal stability, average nucleosomal repeat length, and resistance to micrococcal nuclease digestion are all increased in mature sperm relative to spermatid chromatin. Since the chromatins are identical in histone variant subtypes, the altered physical properties are not a consequence of changes in histone primary structure during spermiogenesis. The data are interpreted to mean that dephosphorylation of the N-terminal regions of Sp H1 and Sp H2B in late spermatid nuclei permits strong ionic binding of these highly basic regions to the extended linker, stabilizing the highly condensed structure of sperm chromatin.

While conservation of histone amino acid sequence and structure is the general rule for eukaryotic chromosomes, many exceptions occur in sperm cells of animal and plant species (Poccia, 1986). In extreme instances, such as protamine-containing sperm of trout and mammals, nucleosomal structure is abandoned, and histones are replaced by various highly basic proteins. While such proteins are often assumed to be involved in chromatin condensation or packing, in most cases little is known about how their structural features contribute to the

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