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Protein 4.1: Its association with the human erythrocyte membrane

(binding analysis/glycophorin A/papain digestion)

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ABSTRACT **^{125}I-labeled protein 4.1a and 4.1b have equal ability to reassociate with inside-out erythrocyte vesicles that were depleted of protein 4.1 in addition to other peripheral membrane proteins. The reassociation of ^{125}I-labeled protein 4.1 to protein 4.1-depleted vesicles at 4°C is salt dependent, pH dependent, and saturable with a K_d of 42–50 nM and an extrapolated maximal binding capacity of 120–140 μg of protein 4.1 bound per mg of vesicle protein or 60–70 μg of protein 4.1 bound per mg of ghost protein, correlating with the protein 4.1 content in the erythrocyte membrane (6–7% of the total membrane protein). Selective proteolytic cleavage of these vesicles with papain (5 μg/ml at 4°C) eliminates >60% of the high-affinity binding sites; therefore, we conclude that the interaction of protein 4.1 with the cytoplasmic membrane surface is through a specific high-affinity protein–protein association.**

Protein 4.1 is an essential component of a fibrous two-dimensional meshwork of proteins located on the cytoplasmic surface of the erythrocyte membrane, which is now commonly referred to as the membrane skeleton. The major polypeptides of the erythrocyte membrane are designated by Steck's nomenclature (1). Protein 4.1 was originally characterized as a peripheral membrane protein of 78,000 molecular weight when analyzed by $NaDodSO_4$/PAGE in continuous buffer systems (1, 2), which was present in ≈200,000 copies on the cytoplasmic surface of the erythrocyte plasma membrane (1). Purified protein 4.1 has been found to bind to the terminal ends of spectrin tetramers and to stimulate the binding of spectrin to actin protofilaments, probably by formation of a spectrin–protein 4.1–actin ternary complex (for reviews, see refs. 3 and 4). That this complex is essential to the structural integrity of the erythrocyte membrane has been suggested by demonstration of defects in the spectrin–protein 4.1–actin interaction in certain kindreds with the hemolytic anemia termed hereditary spherocytosis (5, 6) and with deficiencies in protein 4.1 in a variant of hereditary elliptocytosis (7).

Although protein 4.1 appears as a single component on $NaDodSO_4$/PAGE in continuous buffer systems, two major components of 80,000 and 78,000 daltons are seen when electrophoresis is performed in a discontinuous buffer system (8, 9). These two components, referred to as proteins 4.1a and 4.1b, are sequence-related phosphoproteins that bind spectrin with equal efficiency and are present in ≈100,000 copies per erythrocyte (9). Greater than 90% of the protein 4.1 (a and b) remains associated with the membrane under conditions of low ionic strength, which cause removal of >95% of the spectrin and actin. Furthermore, partial removal of this membrane-associated protein 4.1 from spectrin/actin-depleted vesicles demands high-ionic-strength extractions at 37°C. Therefore, protein 4.1 is believed to have a high-affinity membrane binding site separate from its interactions with spectrin and actin (for reviews, see refs. 3 and 4). In this study, we demonstrate that protein 4.1 (a and b) can be reassociated with high affinity to the cytoplasmic surface of protein 4.1-depleted vesicles (also depleted of spectrin and actin) in a salt- and pH-dependent, saturable manner. The physiological concentration of protein 4.1 can be bound to these vesicles through a specific protein–protein interaction with a single class of high-affinity membrane attachment sites. These data have been presented in preliminary form (10).

MATERIALS AND METHODS

Preparation of ^{125}I-Labeled Protein 4.1 (^{125}I-Protein 4.1). Purification of ^{125}I-protein 4.1 was by the standard method of Tyler *et al.* (11) with our modifications (9).

Protein 4.1-Depleted Inverted Vesicle Preparation. Human erythrocyte ghosts were prepared essentially by the procedure of Dodge *et al.* (12) except that the lysing buffer was 5 mM sodium phosphate/1 mM EDTA/0.4 mM diisopropyl fluorophosphate (iPr_2P-F), pH 7.6. Ghosts were washed once with 0.1 mM EDTA/0.2 mM iPr_2P-F, pH 8.0, and then were incubated with this same buffer at 37°C for 30 min. Spectrin/actin-depleted inverted vesicles were sedimented (39,000 × *g* for 30 min at 4°C) and washed again with 0.1 mM EDTA/0.2 mM iPr_2P-F, pH 8.0 (39,000 × *g* for 30 min at 4°C). In order to remove proteins 2.1 and 4.1 (≈60–80%) from the membrane, vesicles were incubated with 5 mM $NaPO_4$/1 mM EDTA/1 M KCl/0.4 mM iPr_2P-F, pH 7.6, at 37°C for 60 min, sedimented (39,000 × *g* for 30 min at 4°C), and washed twice with the assay buffer (5 mM sodium phosphate/1 mM EDTA/130 mM KCl/10 mM NaCl/2 mM NaN_3/0.2 mM dithiothreitol/1 mg of bovine serum albumin per ml, pH 8.0).

Right-Side-Out Vesicle Preparation. Right-side-out vesicles were prepared as described (13).

Binding Assay. Various concentrations of native and heat-denatured human erythrocyte ^{125}I-protein 4.1 were incubated with protein 4.1-depleted inverted vesicles (15 μg) in (total volume, 0.3 ml) 5 mM sodium phosphate/1 mM EDTA/130 mM KCl/10 mM NaCl/2 mM NaN_3/0.2 mM dithiothreitol/1 mg of bovine serum albumin, pH 8.0, at 4°C for 2 hr. Free and membrane-bound ^{125}I-protein 4.1 were separated by layering 200 μl of this mixture over 200 μl of 15% sucrose in binding buffer in a polyethylene Microfuge tube, followed by centrifugation (39,000 × *g* for 30 min at 4°C). The tubes were then frozen in liquid N_2, and the tips containing bound ^{125}I-protein 4.1 and the tops containing free ^{125}I-protein 4.1 were assayed for radioactivity with a Packard Auto 500 gamma counter. Control samples with heat-denatured ^{125}I-protein 4.1 (20 min at 50°C) were tested in each experimental condition, and these values (<20%) were routinely subtracted from values obtained with the native protein 4.1. Samples containing only ^{125}I-protein 4.1 and no vesicles also were tested at each condition, and the amount of ^{125}I-protein 4.1

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Abbreviations: iPr_2P-F, diisopropylfluorophosphate; PAS, periodic acid/Schiff's reagent stain.

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within the bound fraction was always <2% of the protein 4.1 layered onto the sucrose cushion. Each experimental point was performed either in duplicate or triplicate with a range never exceeding 5% of the experimental value. When right-side-out vesicles were substituted in the assay, the binding curve resembled the curve obtained when heat-denatured protein 4.1 was bound to protein 4.1-depleted vesicles. In the case of right-side-out vesicles, the binding never exceeded 30% of the values obtained when native ^{125}I-protein 4.1 was bound to protein 4.1-depleted inside-out vesicles. We do not use the right-side-out vesicles as our systematic control because the low level of binding is contributed to by: (*i*) the fact that only 70% of the vesicles in this preparation are considered to be right side out by morphological criteria (13), (*ii*) the right-side-out vesicles were not completely resealed, and (*iii*) nonspecific binding occurs to the outside surface, which is the determinant that one would want to be testing.

Proteolysis of Protein 4.1-Depleted Inverted Vesicles. Protein 4.1-depleted inverted vesicles (2 ml at 1 mg/ml) prepared in the absence of iPr_2P-F were incubated for 1 hr at 4°C with and without trypsin (10 μg/ml), α-chymotrypsin (10 μg/ml), or papain (0.1–5 μg/ml) in binding buffer. The proteolysis was stopped by adding an equal volume of binding buffer containing 10 mM iPr_2P-F (trypsin or α-chymotrypsin) or 20 mM Na iodoacetate (papain). Vesicles were washed six times with binding buffer without dithiothreitol (39,000 × *g* for 20 min at 4°C) and then were resuspended with the binding buffer (with dithiothreitol).

Gel Electrophoresis. $NaDodSO_4$/PAGE in 1.5-mm thick slab gels was performed by either the continuous buffer system of Fairbanks *et al.* (2) or the discontinuous buffer system of Laemmli (8).

Protein Determinations. Protein determinations were performed by the Bio-Rad protein assay (14).

RESULTS

Selective Binding of Protein 4.1 (a and b) to Protein 4.1-Depleted Inside-Out Vesicles. We have characterized the binding of purified ^{125}I-protein 4.1 to inside-out erythrocyte vesicles that are depleted of spectrin, actin, syndeins, and ≈80% of their protein 4.1 content by high-ionic-strength extraction of spectrin-depleted inverted vesicles. These protein 4.1-depleted vesicles (Fig. 1, lane B) have been shown to be at least 85% inside out by morphological criteria (15). ^{125}I-protein 4.1, which contains proteins 4.1a and 4.1b in a ratio of 1.2:1 (4, 9), was reassociated with the protein 4.1-depleted vesicles (physiological ionic strength, pH 8.0, at 4°C), and bound and free ^{125}I-protein 4.1 were separated through a sucrose cushion, electrophoresed in a discontinuous buffer system (8), and visualized by autoradiography. Both free and membrane-associated protein 4.1 fractions contained ^{125}I-protein 4.1a and 4.1b in a 1.2:1 ratio (Fig. 1, lanes C and D). Therefore, proteins 4.1a and 4.1b have equal capability to reassociate with protein 4.1-depleted vesicles, and it is reasonable to use purified protein 4.1, which contains a mixture of 4.1a and 4.1b to study the protein 4.1–membrane association.

When a fixed amount of ^{125}I-protein 4.1 (20 μg/ml) was incubated with increasing concentrations of 4.1-depleted vesicles, binding was linear up to ≈20 μg of vesicle protein (Fig. 2). A vesicle protein concentration of 15 μg was chosen for all subsequent experiments with protein 4.1-depleted vesicles. When right-side-out erythrocyte vesicles (>70% right side out) were substituted in the assay, binding was always <30% of the values obtained with protein 4.1-depleted vesicles (data not shown; see *Materials and Methods*). Therefore, protein 4.1 was selectively reassociating with the cytoplasmic membrane surface. The binding was not to residual protein 4.1 remaining on the vesicles because, when vesicles that were depleted of only 60% of their protein 4.1 content were substituted into the assay, binding decreased relative to the 80% depleted vesicles utilized in Fig. 2 (data not shown).

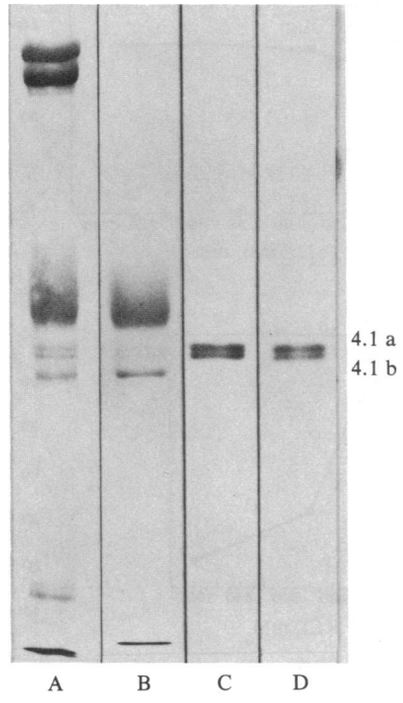

FIG. 1. Binding of proteins 4.1a and 4.1b to protein 4.1-depleted inverted vesicles. ^{125}I-protein 4.1 (20 μg/ml) was incubated with protein 4.1-depleted inverted vesicles (15 μg) at 4°C for 2 hr as described. Free and bound ^{125}I-protein 4.1 were separated by centrifugation (39,000 × *g* for 30 min) and subjected to $NaDodSO_4$/PAGE with the Laemmli buffer system (8) and a 7% polyacrylamide separating gel. Autoradiographs were exposed for 30 min at −20°C using Kodak X-Omat XAR-5 film with a Dupont Cronex Lightning Plus intensifying screen. (*A*) Coomassie blue staining of human erythrocyte membrane protein (64 μg). (*B*) Coomassie blue staining of protein 4.1-depleted inverted vesicles (32 μg). (*C*) Autoradiograph of bound ^{125}I-protein 4.1. (*D*) Autoradiograph of free ^{125}I-protein 4.1.

Optimizing the Protein 4.1–Membrane Reassociation. The time course of ^{125}I-protein 4.1 reassociation (Fig. 3*A*) was relatively rapid, with 60% of maximal binding observed at 6 min and maximal binding reached at 50 min of incubation.

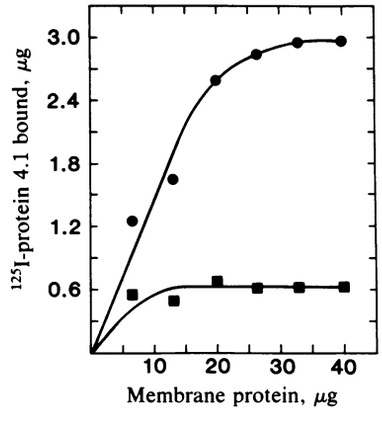

FIG. 2. Dependency of protein 4.1 binding upon vesicle concentration. Binding of native (●) and heat-denatured (■) (50°C for 20 min) ^{125}I-protein 4.1 (130,000 cpm/μg) to various amounts of protein 4.1-depleted vesicles (6–40 μg) at 4°C for 2 hr was as described. There was 4 μg of ^{125}I-protein 4.1 in the 200 μl of analyzed incubation mixture.

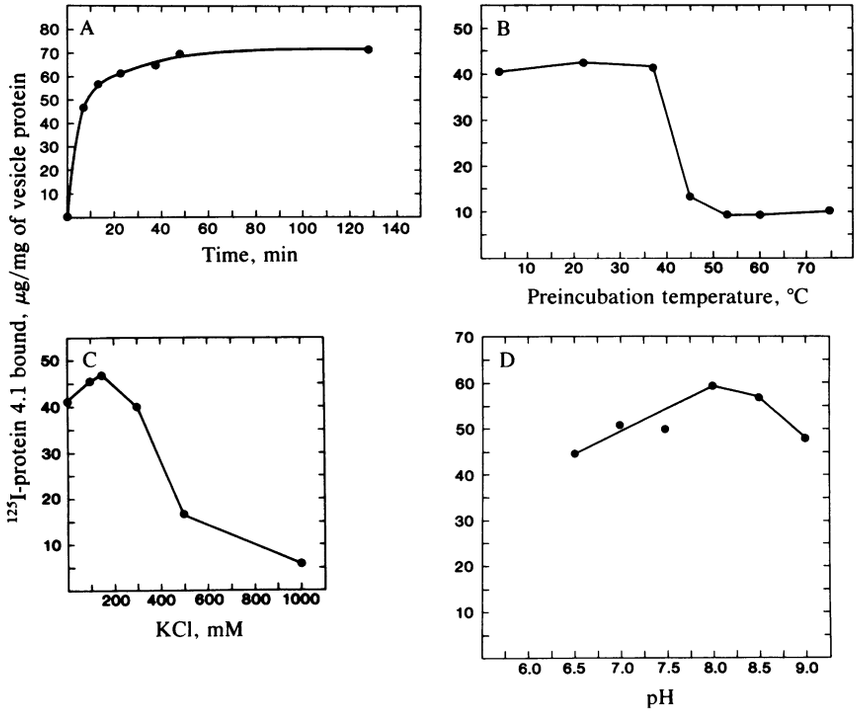

FIG. 3. Optimizing the protein 4.1-membrane reassociation. (*A*) Kinetics of protein 4.1–membrane interaction was determined by incubating ^{125}I-protein 4.1 (2 μg/ml; 232,000 cpm/μg) with protein 4.1-depleted inverted vesicles (2 μg) for various lengths of time. The actual incubation time was assumed to continue until the rotor reached maximal velocity. (*B*) ^{125}I-protein 4.1 (20 μg/ml; 231,000 cpm/μg) was preincubated at various temperatures for 20 min. The ^{125}I-protein 4.1 was cooled to 4°C and incubated with protein 4.1-depleted inverted vesicles (15 μg) at 4°C for 2 hr as described. (*C*) ^{125}I-protein 4.1 (20 μg/ml; 155,000 cpm/μg) was incubated with protein 4.1-depleted inverted vesicles (15 μg) in 5 mM sodium phosphate/1 mM EDTA/10 mM NaCl/1 mM dithiothreitol containing various concentrations of KCl, all brought to a final pH of 8.0. Incubations were for 2 hr at 4°C. (*D*) ^{125}I-protein 4.1 (20 μg/ml; 87,000 cpm/μg) was incubated at 4°C for 2 hr with protein 4.1-depleted inverted vesicles (15 μg) in the assay buffer brought to various final pH values. For all experiments (*A–D*), membrane-bound and free ^{125}I-protein 4.1 were determined as described.

Because the concentration of ^{125}I-protein 4.1 (2 μg/ml) used in this experiment was the minimal concentration used in subsequent experiments, we were assured that a 2-hr incubation would be ample time to bring all subsequent incubations to equilibrium.

Pretreatment of ^{125}I-protein 4.1 at various temperatures for 20 min prior to incubation with protein 4.1-depleted vesicles showed that a loss of binding capacity occurred abruptly between 37°C and 45°C (Fig. 3*B*). Above 50°C, binding was decreased by ≈80%; therefore, this was the condition used for our subsequent heat-denatured controls.

The protein 4.1–membrane association depended critically on the salt concentration of the incubation medium (Fig. 3*C*). ^{125}I-protein 4.1 was incubated with protein 4.1-depleted vesicles in an incubation medium containing 5 mM sodium phosphate, 1 mM EDTA, 10 mM NaCl, 1 mM dithiothreitol, and various concentrations of KCl from 0 to 1 M, all brought to a fixed pH of 8.0. Maximal binding was observed at 130 mM KCl, being reduced to 36% of maximum at 500 mM KCl and 16% of maximum at 1 M KCl. These values are consistent with the requirement of >500 mM KCl to disassociate protein 4.1 from spectrin-depleted vesicles. A broad pH optimum was observed for the protein 4.1–membrane reassociation, with maximal binding observed at pH 8.0 (Fig. 3*D*).

Protein 4.1 Interacts with the Membrane Through a Specific High-Affinity Protein–Protein Association. Various concentrations of ^{125}I-protein 4.1 (5–55 μg/ml) were incubated under the optimal salt and pH conditions described above with protein 4.1-depleted vesicles (15 μg) for 2 hr at 4°C, and saturable binding was observed (see binding isotherm, Fig. 4*A*). Scatchard analysis of these data yielded a linear plot with a K_d of 42–50 nM (range of five experiments) and an extrapolated maximal binding capacity of 120–140 μg of protein 4.1 bound per mg of vesicle protein (Fig. 4*B*). Because protein 4.1-depleted vesicles contained ≈50% of the total membrane protein of erythrocyte ghosts, our experimental binding capacity was equal to 60–70 μg of protein 4.1 bound per mg of ghost protein. This value is equivalent to the amount of protein 4.1 in the native erythrocyte membrane, where it constitutes 6–7% of the total protein (3, 4). Therefore, the number of high-affinity association sites should approximate the number of protein 4.1 copies per ghost, which would be

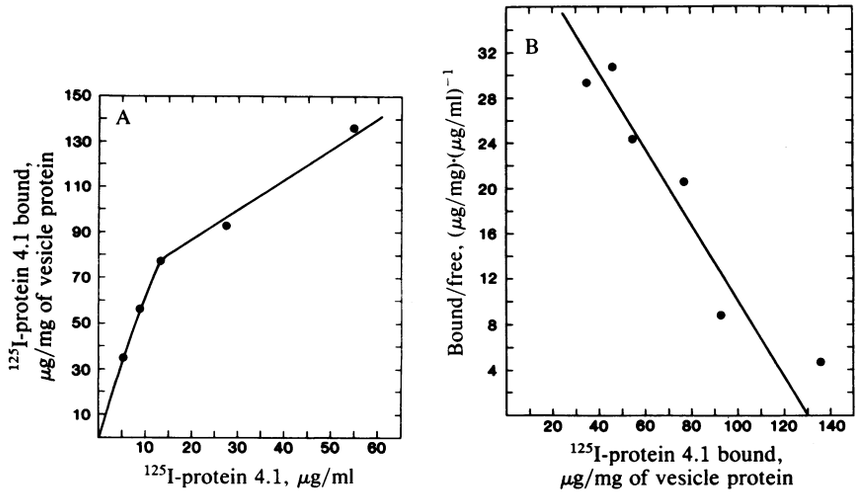

FIG. 4. Binding of various concentrations of ^{125}I-protein 4.1 to a fixed concentration of protein 4.1-depleted inverted vesicles under the optimal binding conditions obtained in Fig. 3 and described in *Methods*. (*A*) Various concentrations of ^{125}I-protein 4.1 (439,000 cpm/μg) were incubated with 4.1-depleted inverted vesicles (15 μg) at 4°C for 2 hr. (*B*) Scatchard analysis of data from *A*.

≈200,000 attachment sites. The labeling of protein 4.1 with ^{125}I-labeled Bolton–Hunter reagent did not appear to alter its binding characteristics, as we used ^{125}I-protein 4.1 ranging in specific activity from 8×10^4 to 5×10^5 cpm/μg and obtained identical results.

If the high-affinity protein 4.1-membrane attachment site were a protein, then we should have the ability to eliminate the association by proteolytic digestion of protein 4.1-depleted vesicles. Protein 4.1-depleted vesicles were digested with trypsin (10 μg/ml), chymotrypsin (10 μg/ml), or papain (0.1–5 μg/ml) at 4°C for 1 hr, followed by addition of 10 mM iPr_2P-F (trypsin or α-chymotrypsin) or 20 mM Na iodoacetate (papain) to stop the digestions and by thorough washing. Proteolytic digestion of protein 4.1-depleted vesicles with trypsin and chymotrypsin converted band 3 into a 52,000-dalton membrane-associated fragment (Fig. 5, and refs. 15 and 16), while releasing the 43,000-dalton cytoplasmic domain of band 3 (data not shown; see refs. 15 and 16). Trypsin and chymotrypsin digestion did not affect periodic acid/Schiff reagent (PAS)-stained components designated PAS 1, 2, 3, or 4 but caused partial digestion of another major component of the protein 4.1-depleted vesicles, band 4.2. These digestions with trypsin and chymotrypsin had no effect upon the binding of protein 4.1 to 4.1-depleted vesicles (Table 1). Papain, as previously demonstrated (17), cleaved band 3 into a 52,000-dalton membrane-associated fragment, destroyed band 4.2, and led to a loss of the PAS 1 band (glycophorin A) with a reciprocal appearance of PAS-stainable material traveling with faster electrophoretic mobility (Fig. 5). Increasing concentrations of papain used for digestion of protein 4.1-depleted vesicles caused a corresponding reduction in the binding of ^{125}I-protein 4.1 to the cytoplasmic membrane surface. At 5 μg of papain per ml, where ≈67% of PAS 1 had been digested (Fig. 5), a reduction in protein 4.1 binding of 61% was observed. The effect of papain upon the protein 4.1–membrane interaction was not due to residual protease activity, as autoradiographs of NaDodSO$_4$/PAGE of the bound and free fractions of ^{125}I-protein 4.1 incubated with papain-treated vesicles were identical to untreated controls (data not shown). Therefore, the reduction in the reassociation of protein 4.1 with papain-treated inverted vesicles was due to selective release or destruction of the cytoplasmic portion of a protein that contains the high-affinity binding site for protein 4.1.

Table 1. Summary of proteolytic digestion experiments

Protease	Protease, μg/ml	Protein remaining on vesicles, %	Reduction in protein 4.1 binding, %
Trypsin	10	61	0
α-Chymotrypsin	10	44	2
Papain	0.1	95	7
	1	42	44
	3	38	52
	5	31	61

Data were from experiments in which ^{125}I-protein 4.1 at 20 μg/ml was incubated with protein 4.1-depleted inverted vesicles (15 μg equivalents), and membrane-bound ^{125}I-protein 4.1 was determined; 1 μg equivalent is the amount of vesicle protein remaining when 1 μg of protein 4.1-depleted vesicles is proteolytically digested. The control value for binding of ^{125}I-protein 4.1 to undigested protein 4.1-depleted vesicles was 55.5 μg of protein 4.1 bound per mg of vesicle protein.

DISCUSSION

We have established a method for studying the protein 4.1–membrane interaction, which should be useful in screening hereditary spherocytosis, elliptocytosis, and pyropoikilocytosis blood samples for potential defects. By utilizing this

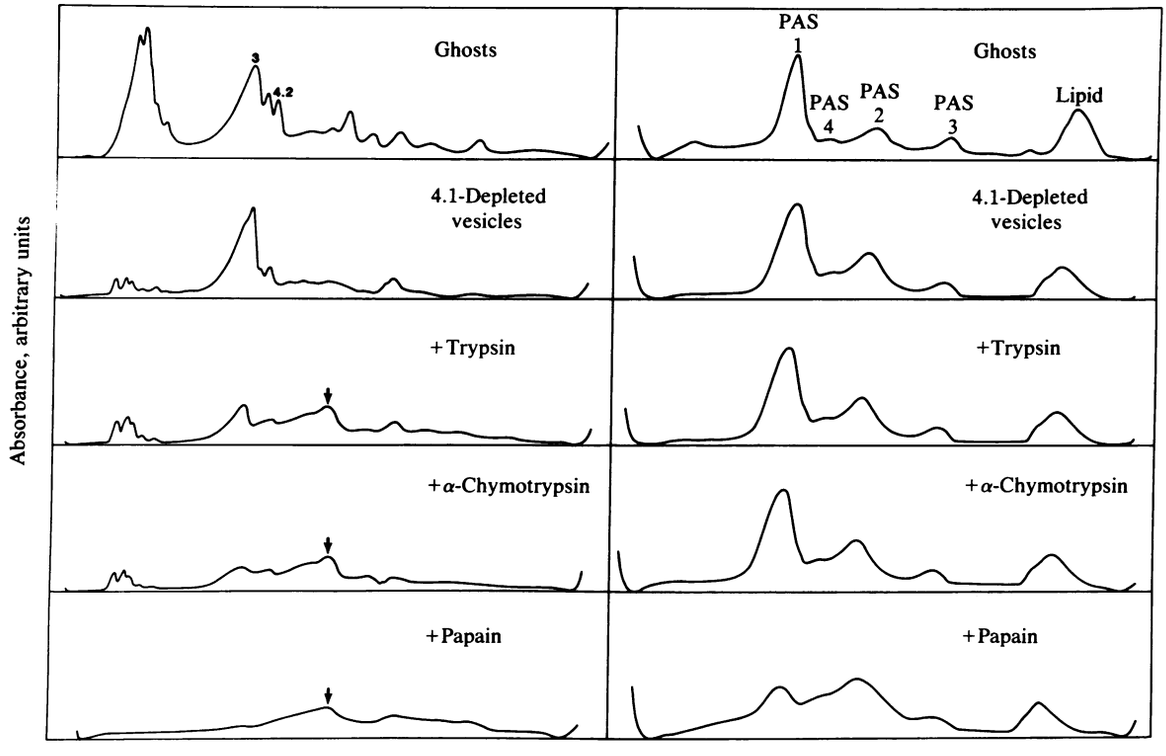

FIG. 5. Digestion of protein 4.1-depleted inverted vesicles with trypsin, chymotrypsin, and papain. Human erythrocyte ghost protein, protein 4.1-depleted vesicles, and trypsin (10 μg/ml)-, chymotrypsin (10 μg/ml)-, or papain (5 μg/ml)-treated vesicles were analyzed by NaDodSO$_4$/PAGE as described (2). Duplicate samples run on two lanes were stained either with Coomassie blue or PAS as described (2). Gel lanes were scanned with an LKB Zeineh Lazer scanning densitometer using the red laser source for Coomassie-stained protein and the tungsten light source for PAS-stained protein. The arrows designate the 52,000-dalton membrane-associated fragment of band 3.

technique, we have demonstrated that protein 4.1 interacts in a salt- and pH-dependent, saturable manner with protein 4.1-depleted inverted vesicles. Failure of one group to obtain saturable binding of protein 4.1 to inverted vesicles (18) in previous studies was probably due to several factors including nonoptimal assay conditions; performance of the assay at 25°C, which enhances low-affinity protein 4.1-membrane interactions; and utilization of protein 4.1-depleted vesicles prepared in the presence of 1 M KCl and 0.4 M urea, conditions that may cause partial denaturation of high-affinity attachment sites.

Over the past 3 years, it has been suggested that the protein 4.1 binding site is (*i*) a relatively minor sialoglycoprotein, glycophorin C (19); (*ii*) the major sialoglycoprotein, glycophorin A (20); and (*iii*) phosphatidylserine domains (21). Obviously, the question of how protein 4.1 is attached to the membrane remains unresolved. We previously reviewed experimental and theoretical problems with the above mentioned studies (3, 4) and, therefore, will not reiterate those difficulties here.

Our finding that papain digestion of the protein 4.1-depleted inverted vesicles eliminates the high-affinity protein 4.1–membrane association suggests that protein 4.1 binds to the membrane through a specific protein–protein interaction. This eliminates the possibility that phosphatidylserine domains represent the high-affinity protein 4.1 binding sites (21), although it is still possible that lower-affinity biologically significant interactions between protein 4.1 and lipids may exist. Because the attachment site is a protein present at ≈200,000 copies, the only proteins that appear in large enough quantity on the cytoplasmic surface of the protein 4.1-depleted vesicles to represent reasonable candidates would be glycophorin A, band 3, and band 4.2. Our demonstration that trypsin and chymotrypsin, which release the 43,000-dalton cytoplasmic domain of band 3, do not affect protein 4.1 binding to inverted vesicles clearly demonstrates that this domain of band 3 does not represent the high-affinity protein 4.1 attachment site. We have not ruled out the possibility that the 52,000-dalton membrane-associated tryptic or chymotryptic fragment, which represents the extracellular and intramembranous portions of band 3, might have small segments exposed on the cytoplasmic membrane surface that could serve as the protein 4.1 attachment site. Furthermore, band 4.2, which is partially cleaved by trypsin and chymotrypsin, by the same logic is also not eliminated as a candidate because an active protein 4.1 binding fragment of band 4.2 might still be retained by the membrane.

An appealing candidate for the protein 4.1 attachment site from these studies would appear to be glycophorin A, which is present in ≈200,000 dimers per erythrocyte membrane. Trypsin and chymotrypsin, which do not cleave glycophorin A in protein 4.1-depleted vesicles, have no effect upon ^{125}I-protein 4.1 binding, whereas papain, which cleaves glycophorin A on the cytoplasmic surface of these vesicles, causes a corresponding reduction in binding. However, we must be cautious in suggesting a protein 4.1–glycophorin A interaction for at least two theoretical reasons. First, if glycophorin A represents the high-affinity association site for protein 4.1, it is not clear why glycophorin A does not remain associated with the erythrocyte Triton X-100-extracted membrane skeleton, which retains protein 4.1 (3, 4). Second, subjects with En(a-) erythrocytes completely lack membrane-associated glycophorin A, yet the erythrocytes maintain normal shape, function, and circulatory life span (for a review, see ref. 22). In addition to these unresolved theoretical problems, we must be extremely wary of interpreting some of the simpler approaches being used to test for a potential protein 4.1–glycophorin A association. Glycophorin A is isolated in the presence of neutral detergents (23); removal of these detergents causes aggregation of glycophorin A, and the use of this aggregated glycophorin A in protein 4.1 competition binding studies is likely to indicate a false-positive result due to nonspecific hydrophobic interactions between the glycophorin A aggregates and protein 4.1 (24). Furthermore, the use of glycophorin A antibodies to eliminate binding of ^{125}I-protein 4.1 to protein 4.1-depleted vesicles is difficult to interpret (24), as glycophorin A appears to be associated with band 3 in the membrane (25), and band 3 contains the band 4.2 binding site (26). Therefore, any effect of a glycophorin A antibody could be due to direct blockage of an attachment site on glycophorin A or indirect blockage of an attachment site on band 3 or band 4.2. The identity of the high-affinity protein 4.1 attachment site must still be considered unresolved for the reasons stated above.

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