Modulation of Band 3-Ankyrin Interaction by Protein 4.1

FUNCTIONAL IMPLICATIONS IN REGULATION OF ERYTHROCYTE MEMBRANE MECHANICAL PROPERTIES

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Protein 4.1 is an important structural component of the erythrocyte membrane. In contrast to our detailed understanding of the role of protein 4.1 in regulating membrane mechanical properties through modulation of spectrin-actin interaction, very little is known regarding the functional implications of protein 4.1 interaction with band 3. In the present study, we explored the potential role of protein 4.1-band 3 interaction in modulating membrane mechanical properties. Based on recent studies which identified the sequence motif IRRRY in band 3 as the protein 4.1 interacting domain, we studied the functional consequences of specific dissociation of band 3-protein 4.1 interaction by the synthetic peptide IRRRY. We show that protein 4.1 bound to the inside-out vesicles could be dissociated from band 3 but not from glycophorin C by IRRRY. Furthermore, incorporation of IRRRY into resealed ghosts resulted in decreased membrane deformability and increased membrane mechanical stability. The observed alterations in membrane properties appears to result from increased band 3-ankyrin interaction following dissociation of protein 4.1 from band 3. These studies have enabled us to identify an important functional role for band 3-protein 4.1 interaction in modulating erythrocyte membrane properties.

Mechanical properties of the human erythrocyte membrane are primarily regulated by the spectrin-based membrane skeleton that underlies the lipid bilayer and by membrane proteins that anchor the skeleton to the bilayer (for review, see Ref. 1). Spectrin, actin, protein 4.1, adducin, tropomyosin, tropomodulin, dematin, and p55 are the principal constituents of the membrane skeleton. Lateral interactions among these proteins constitute the composite structure designated as the membrane skeletal network. This network is anchored to the bilayer through vertical interactions, one involving β-spectrin, ankyrin, and band 3, and the other through an interaction between protein 4.1 and glycophorin C (for review, see Refs. 2 and 3).

Recent biochemical studies of the purified protein, together with molecular cloning and sequencing of the 4.1 cDNA, have facilitated construction of a structure and functional map of erythrocyte protein 4.1 molecule (4). Four major structural domains of protein 4.1 with apparent molecular masses of 30, 16, 10, and 22–24 kDa were identified (5). Purified erythrocyte protein 4.1 has been shown to bind with high affinity to spectrin and with lower affinity to actin through its 10-kDa domain (6–10). Protein 4.1 interacts with integral membrane proteins band 3 (11–14) and glycophorin C (15–18) through its 30-kDa domain.

Direct evidence for a critical role for protein 4.1 in maintaining membrane mechanical stability was demonstrated by studies in which normal membrane mechanical stability was restored to the unstable protein 4.1-deficient erythrocyte membranes through incorporation of either purified protein 4.1 (19) or the 10-kDa spectrin-actin binding domain of protein 4.1 (20). In contrast to our detailed understanding of the role of protein 4.1 in regulating membrane mechanical properties through modulation of spectrin-actin interactions, very little is known regarding the functional implications of protein 4.1 interaction with band 3.

In the present study, we explored the potential role of protein 4.1-band 3 interaction in modulating membrane mechanical properties by studying the functional consequences of specific dissociation of band 3-protein 4.1 interaction using the synthetic peptide IRRRY (21). We were able to show that protein 4.1 bound to the inside-out vesicles (IOVs) could be dissociated from band 3 but not from glycophorin C by IRRRY. Furthermore, incorporation of IRRRY into resealed ghosts resulted in decreased membrane deformability and increased membrane mechanical stability. The observed alterations in membrane properties appear to result from increased band 3-ankyrin interaction following dissociation of protein 4.1 from band 3. These studies have enabled us to identify an important functional role for band 3-protein 4.1 interaction in modulating erythrocyte membrane properties.

EXPERIMENTAL PROCEDURES

Materials

After obtaining informed consent, human venous blood was freshly drawn from healthy volunteers. Various synthetic peptides were synthesized by the Fmoc (N-(9-fluorenylmethoxycarbonyl) method and purified by reverse phase high performance liquid chromatography (24). Trypsin (type 1 from bovine pancreas) and trypsin inhibitor (type 1-S from soybean) were purchased from Sigma. Protein 4.1 and ankyrin were purified according to the method developed by Tyler et al. (6) with minor modifications. These purified proteins were labeled using Bolton-Hunter reagent (200 Bq/mmol, DuPont NEN) and were dialyzed

1 The abbreviations used are: IOV, inside-out vesicle; pH 11 IOVs, alkaline-treated inside-out vesicles; T-pH 11 IOVs, trypsin-digested pH 11 inside-out vesicles; DI, deformability index.
against 100 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 5 mM sodium phosphate, pH 7.4, 1 mM NaN₃, and 1 mg/ml bovine serum albumin (the binding buffer).

**Methods**

**Preparation of IOVs—**Inside-out vesicles depleted of all peripheral proteins (pH 11 IOV) and trypsin-digested pH 11 IOV (T-pH 11 IOV) were prepared according to methods described by Danilov et al. (12) with minor modifications. SDS-polyacrylamide gel electrophoresis analysis of T-pH 11 IOVs showed that the cytoplasmic domain of band 3 was completely digested in this IOV preparation. In contrast, the cytoplasmic domain of glycophorin C remained intact.

**Measurement of ¹²⁵I-Protein 4.1 Bound to IOVs—**¹²⁵I-protein 4.1 (330 µg) was incubated separately with pH 11 IOV (300 µg) and T-pH 11 IOV (300 µg) in 1 ml of the binding buffer for 1 h at 24 °C. IOVs were collected by centrifugation and washed three times to remove unbound ¹²⁵I-protein 4.1. Peptides IRRRY or FGGLVRD at various concentrations were added to ¹²⁵I-protein 4.1-reconstituted IOVs (30 µg) and incubated at 37 °C for 40 min. Following incubation, the mixture was layered onto 200 µl of 8% sucrose cushion in binding buffer and centrifuged at 12500 × g for 30 min at 4 °C. IOVs were collected and the amount of ¹²⁵I-protein 4.1 bound to IOVs was quantitated using a gamma counter. Amount of ¹²⁵I-protein 4.1 bound to denatured IOVs was determined to quantitate nonspecific binding. Nonspecific binding constituted approximately 3% of total binding and was subtracted from the measured values to derive the specific binding. Data shown are the mean of triplicate measurements.

**Reconstitution of Erythrocyte Ghosts with Peptides—**Washed erythrocytes were lysed in 35 volumes of hypotonic buffer (5 mM Tris, 5 mM KCl, pH 7.4) at 4 °C and washed four times. The white membranes were incubated at 37 °C for 10 min with synthetic peptides at various concentrations with gentle stirring. A small volume of a mixture of KCl, MgCl₂, and dithiothreitol and trypsin inhibitor was added to the membrane suspensions to obtain final concentrations of 150 mM, 1 mM, and 1 mM and 1.7 mg/ml, respectively, and the ghost suspensions incubated at 37 °C for 40 min to allow membrane resealing (25).

**Measurement of Membrane Mechanical Properties—**The resealed ghosts were suspended in 45% dextran, and membrane mechanical stability was quantitated using an ektacytometer as described previously (25, 26). The rate of decrease of deformability index (DI) at a constant applied shear stress of 750 dynes/cm² was analyzed to quantify membrane mechanical stability (25, 26). To measure membrane deformability, resealed ghosts were suspended in Stractan (22 centipoise viscosity; 290 mosm) and exposed to an increasing shear stress (0–150 dynes/cm²) in the ektacytometer. DI versus applied shear stress curve was analyzed to quantify membrane deformability (26).

**Measurement of ¹²⁵I-Ankyrin Binding to pH 11 IOVs—**Binding of ¹²⁵I-ankyrin to the cytoplasmic domain of band 3 was quantitated according to the method described by Bennett et al. (22). Briefly, ¹²⁵I-ankyrin at various concentrations was incubated with pH 11 IOVs or pH T-pH 11 IOVs preincubated with protein 4.1 in the binding buffer at 24 °C for 3 h. The mixture was layered onto 8% sucrose cushion, centrifuged at 4 °C, IOVs collected, and the amount of bound ¹²⁵I-ankyrin determined using a gamma counter.

**RESULTS**

**Dissociation of Membrane-bound Protein 4.1 by IRRRY—**To document that the synthetic peptide IRRRY can selectively dissociate protein 4.1 from band 3 without affecting protein 4.1-glycophorin C interaction, pH 11 IOVs and T-pH 11 IOVs were first reconstituted with ¹²⁵I-protein 4.1 and subsequently incubated with increasing concentrations of the peptide, and the release of bound protein 4.1 was monitored (Fig. 1).

194 µg of protein 4.1/mg of vesicle proteins bound to band 3 and glycophorin C on pH 11 IOVs, while 98 µg of protein 4.1/mg of vesicle proteins bound to glycophorin C on T-pH 11 IOVs. These measured values are in excellent agreement with results previously reported for protein 4.1 binding to IOVs (11–14). Incubation with increasing concentrations of IRRRY resulted in a dose-dependent displacement of bound protein 4.1 from pH 11 IOVs (Fig. 1). IRRRY incorporated with protein 4.1, only 100 µg of protein 4.1/mg of vesicle proteins remained associated with the pH 11 IOVs compared to 194 µg of protein 4.1/mg of vesicle proteins associated with IOVs in the absence of the peptide. In marked contrast, IRRRY did not displace bound protein 4.1 from T-pH 11 IOVs (Fig. 1). The finding that IRRRY decreased the amount of protein 4.1 bound to pH 11 IOVs to the same levels as that found normally bound to T-pH 11 IOV (98 µg of protein 4.1/mg of vesicle proteins) implies that IRRRY specifically dissociated ¹²⁵I-protein 4.1 bound to band 3 but has no effect on protein 4.1 binding to glycophorin C. In contrast to IRRRY, the control peptide FGGLVRD did not release bound protein 4.1 from pH 11 IOVs (data not shown). To further validate the specificity of IRRRY in regulating band 3–protein 4.1 interaction, the ability of this peptide to dissociate ankyrin from band 3 was assessed. In contrast to our findings with protein 4.1 reconstituted vesicles, IRRRY was not able to release ¹²⁵I-ankyrin from band 3 in ankyrin reconstituted pH 11 IOVs (data not shown).

**Effect of IRRRY and Other Synthetic Peptides on Membrane Mechanical Properties—**To determine the functional consequences of dissociation of band 3–protein 4.1 interaction, membrane deformability and mechanical stability of erythrocyte ghosts reconstituted with different synthetic peptides were measured. Representative data for membrane mechanical stability of ghosts prepared in the presence and absence of the peptide IRRRY are shown in Fig. 2A. IRRRY caused a dose-dependent increase in membrane mechanical stability as revealed by slower rates of decline in the deformability index of peptide reconstituted ghosts compared to control ghosts. Membranes of ghosts reconstituted with IRRRY at 2 mM were 1.5 times more stable than membranes of control ghosts, while ghosts reconstituted with IRRRY at 5 mM were 2.6 times more stable. IRRRY incorporation into ghosts also resulted in decreased membrane deformability (Fig. 2B) as revealed by the higher levels of applied shear stress needed to produce an equivalent increase in deformability index of these ghosts compared to control ghosts. Membrane deformability of ghosts reconstituted with IRRRY at 2 mM was decreased 2-fold, while deformability of ghosts reconstituted with IRRRY at 5 mM decreased 3.2-fold. Neither membrane mechanical stability nor
membrane deformability was altered following resealing of the ghosts with the control peptide FGGLVRD (data not shown). Incorporation of either of the peptides also had no effect on shape of resealed ghosts (data not shown).

To confirm that entrapment of IRRRY into the ghosts is essential for its observed effects on membrane mechanical properties, IRRRY was added to resealed ghosts so that the peptide could have access only to the outer surface of the membranes and not to the cytoplasmic domain of band 3. Addition of 6 mM IRRRY to resealed ghosts did not induce changes in either membrane deformability and of increase in mechanical stability were dependent on concentrations of IRRRY incorporated into ghosts.

mediating membrane changes, we evaluated the effect of a number of additional peptides on membrane mechanical properties. In addition to IRRRY, peptides YRRRI and IRRRI also induced decreases in membrane deformability and increases in mechanical stability (data not shown). In contrast, peptides IRLRY and IRARY did not affect either deformability or mechanical stability of resealed membranes.

Effect of Protein 4.1 on Ankyrin Binding to IOVs—To determine whether the IRRRY-induced functional changes in erythrocyte membrane are the result of changes in the affinity of band 3-ankyrin interaction following dissociation of protein 4.1 from band 3 we quantitated ankyrin binding to native pH 11 IOVs and pH 11 IOVs previously preincubated with various concentrations of protein 4.1 (Fig. 3A). Scatchard analysis of ankyrin binding (Fig. 3B) to native IOVs showed high ($K_d = 4.94 \times 10^{-8}$ M) and low ($K_d = 10.8 \times 10^{-8}$ M) affinity phases of association in the absence of protein 4.1 (Table I), consistent with the data of Bennett et al. (22), Davis and Bennett (23), and Low et al. (27). The total binding capacity of ankyrin to band 3
was 181 μg/mg of vesicle proteins corresponding to a molar ratio of one ankyrin per five to six band 3 monomers. Based on extrapolation of the Scatchard plot in Fig. 3B, the binding capacities of high and low affinity binding sites were estimated to be 72 and 109 μg/mg, respectively. When band 3 on pH 11 IOV was previously half-saturated with protein 4.1, the binding capacity of high affinity sites decreased to 44 μg/mg, while the total binding capacity remained unchanged (Table I) through an increase in binding capacity contributed by low affinity binding sites. Complete saturation of band 3 with protein 4.1 resulted in almost a complete loss of ankyrin binding to band 3 through the high affinity binding sites (16 μg/mg), while the total binding capacity once again remained unchanged (Table I). Thus while protein 4.1 binding to band 3 had little or no effect on the total binding capacity of ankyrin to band 3, it had significant effect on relative contributions of high and low affinity sites for ankyrin binding. Protein 4.1 binding to band 3 thus appears to play a significant role in modulating the affinities of ankyrin association with band 3.

**DISCUSSION**

An important structural role for protein 4.1 in regulating erythrocyte membrane properties through its interaction with spectrin and actin has been previously established. Biochemical studies have documented that in addition to its interactions with spectrin and actin, protein 4.1 also interacts with p55, calmodulin, glycoporphin C, and band 3. However, the functional consequences of these other interactions of protein 4.1 have not been well delineated. The present study enabled us to document a hitherto unrecognized role for protein 4.1 in regulating membrane mechanical properties through modulation of the ankyrin-band 3 interaction.

Jons and Drenckhahn (21) showed that arginine-rich cluster IRRRY in the cytoplasmic domain of band 3 serves as the major binding site for protein 4.1. Their study indicated that the binding of protein 4.1 to band 3 and to IOVs can be inhibited by synthetic peptides IRRRY and FGGLVDRIRRYY but not by the peptide FGGLVRD. Based on these findings, we explored the use of synthetic peptide IRRRY to dissociate protein 4.1 from band 3 and determining the functional consequences of modifying band 3-protein 4.1 interaction. IRRRY indeed selectively dissociated protein 4.1 from band 3 but had no effect on protein 4.1 interaction with glycoporphin C or on interaction of ankyrin with band 3. IRRRY induced dissociation of protein 4.1 from band 3 resulted in marked alterations in membrane mechanical properties. Furthermore, our finding that peptides YRRRI and IRRRY induced membrane alteration similar to IRRRY, while peptides IRLRY and IRARY had no effect on membrane properties suggests that the sequence motif IRRRY may be critical for mediating the interaction of cytoplasmic domain of band 3 with protein 4.1.

Our finding that 50% of total protein 4.1 bound to IOVs is displaced by IRRRY is consistent with previous reports which showed that 50% of membrane-bound protein 4.1 is linked to band 3 (11–14). These findings, however, are at variance with the findings of Hemming et al. (17), who reported using a solid phase binding assay that only 20% of protein 4.1 is bound to band 3. Our finding that IRRRY is able to specifically displace 50% of membrane-bound protein 4.1 from normal IOVs (binding to both band 3 and glycoporphin C) but has no effect of membrane-bound protein 4.1 in tryptic-treated IOVs (binding only to glycoporphin C) implies that band 3 indeed accounts for half of membrane-bound protein 4.1.

The functional data on membrane properties we have outlined clearly show that dissociation of protein 4.1 from band 3 induces marked decreases in membrane deformability and marked increases in membrane mechanical stability. As band 3-ankyrin interaction plays an important role in regulating membrane mechanical properties (28) and band 3 can bind to both ankyrin and protein 4.1, we explored the possibility that alterations in band 3-ankyrin interaction following dissociation of protein 4.1 from band 3 may be responsible for observed membrane functional changes. We could indeed document significant differences in the binding of ankyrin to band 3 in the presence or absence of protein 4.1. In the absence of protein 4.1, ankyrin associates with both high and low affinity sites on band 3, consistent with previous findings of Bennett et al. (22), Davis and Bennett (23), and Low et al. (27). Either different oligomeric states or different conformations of band 3 is responsible for both classes of ankyrin binding sites. In fact, Michael and Bennett (29) have recently shown that the interaction of ankyrin with erythrocyte membranes has negative cooperativity. One possible explanation for our finding that binding of protein 4.1 to band 3 induces a marked decrease in the contribution of high affinity binding sites could be that protein 4.1 binding induces conformational changes in band 3, resulting in the transformation of high affinity binding sites to low affinity sites. The alternative possibility is that cooperativity of ankyrin binding to band 3 was abolished by protein 4.1 binding to band 3.

### Table 1: Effect of protein 4.1 on ankyrin binding to IOV

<table>
<thead>
<tr>
<th>Protein 4.1/IOV</th>
<th>$K_d$ ($\times 10^{-5}$ M)</th>
<th>Binding capacity</th>
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<tr>
<td></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>0</td>
<td>4.94</td>
<td>10.8</td>
</tr>
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<tr>
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<td>4.05</td>
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The binding of protein 4.1 to IOVs was previously half-saturated with protein 4.1, the binding capacity of high affinity sites decreased to 44 μg/mg, while the total binding capacity of ankyrin to band 3 was abolished by protein 4.1 binding to band 3. Our finding that IRRRY is able to specifically displace 50% of membrane-bound protein 4.1 from normal IOVs (binding to both band 3 and glycoporphin C) but has no effect of membrane-bound protein 4.1 in tryptic-treated IOVs (binding only to glycoporphin C) implies that band 3 indeed accounts for half of membrane-bound protein 4.1.
the skeletal network. This increased association restricts the ability of the spectrin tetramers to uncoil and extend during induced deformation resulting in decreased membrane deformability and increased membrane mechanical stability. These results further confirm an important role for band 3-ankyrin interaction in regulation of membrane mechanical properties.

Previous studies provided significant insights into the important contribution of protein 4.1 in regulating erythrocyte membrane function through its interaction with skeletal proteins spectrin and actin. The present study shows an additional role for protein 4.1 in modulating membrane function by regulating ankyrin-band 3 interactions. These results also raise the possibility that protein 4.1, which is widely expressed in various cells, may regulate the organization and function of other ankyrin-linked integral membrane proteins.

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