Regulation of CD44-Protein 4.1 Interaction by Ca\textsuperscript{2+} and Calmodulin

IMPLICATIONS FOR MODULATION OF CD44-ANKYRIN INTERACTION\textsuperscript{*}

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Erythrocyte membrane skeletal protein 4.1 isoforms have been identified in a variety of non-erythroid cells. However, interactions between protein 4.1 and its binding partners in non-erythroid cell membranes are poorly understood. In the erythrocyte membrane, protein 4.1 binds to the cytoplasmic domain of band 3 and, through this interaction, modulates ankyrin binding to band 3. The sequences LRRRY or IRRRY in band 3 mediate the interaction between band 3 and protein 4.1. The cytoplasmic domain of CD44, a transmembrane glycoprotein found in erythroid as well as non-erythroid cells, has internal sequences SRRRC and QKKKL. We wanted to determine if protein 4.1 binds to CD44 in a fashion analogous to its binding to band 3 and through this interaction modulates ankyrin binding to CD44. We report here that protein 4.1 binds to the cytoplasmic domain of CD44 with a dissociation constant on the order of 10\textsuperscript{-7} M and that Ca\textsuperscript{2+} and calmodulin reduce the affinity of this interaction. Furthermore, although independent binding of both protein 4.1 and ankyrin to CD44 could be documented, binding of protein 4.1 prevented subsequent ankyrin binding. These studies have enabled us to identify a potentially important functional role for protein 4.1 in modulating ankyrin binding to CD44.

Homologs of human erythrocyte membrane structural proteins such as protein 4.1, ankyrin, and spectrin are present in a variety of non-erythroid cells including human keratinocytes (1–5). However, the identity of membrane proteins with which these structural proteins interact in non-erythroid cells and factors that modulate these protein-protein interactions have not been well defined. In contrast, we have a detailed understanding of the interactions between protein 4.1 and ankyrin with membrane proteins in erythrocyte membrane and the functional consequences of these interactions (6, 7). Mechanical properties of the human erythrocyte membrane are 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. 7). 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 lipid bilayer through vertical interactions, one involving \(\beta\)-spectrin, ankyrin, and band 3, and the other involving protein 4.1 and glycophorin C (for review, see Refs. 6 and 7).

Protein 4.1, in addition to binding glycophorin C, also binds to the cytoplasmic domain of band 3 (8–12) and through this interaction modulates the band 3-ankyrin interaction (13). Furthermore, binding of Ca\textsuperscript{2+} and calmodulin to protein 4.1 reduces the affinity of its interactions with membrane proteins (12). Protein 4.1 could thus play a critical role in modulating interactions of the spectrin-based network with the plasma membrane and, through these induced dynamic changes in protein-protein interactions, regulate membrane function (13).

Recent biochemical and biophysical studies have facilitated mapping of erythrocyte 4.1 functions relative to its molecular structure (for review, see Ref. 8). Four major structural domains of protein 4.1 with apparent molecular masses of 30, 16, 10, and 22–24 kDa were identified. Protein 4.1 interacts with integral membrane proteins glycophorin C and band 3 through its 30-kDa domain (12, 14–16) and with spectrin and actin through its 10-kDa domain (17, 18). Jons and Drenckhahn (9) have identified the sequence motifs LRRRY and IRRRY in the cytoplasmic domain of band 3 to be involved in binding to the sequence motif LDEEDY in the 30-kDa domain of protein 4.1. Although neither band 3 nor glycophorin C is present in most non-erythroid cells, these cells do contain adhesion molecules such as cadherins and CD44 (19), which have the potential to interact with the cytoskeletal components. In fact, ankyrin has been shown to associate with CD44 in T-lymphoma cells (20). However, there is no evidence for a direct interaction between protein 4.1 and CD44.

Because the cytoplasmic domain of CD44, a transmembrane glycoprotein, has internal sequences SRRRC and QKKKL, we wanted to determine if protein 4.1 can bind to CD44 in a fashion analogous to its binding to band 3 and, through this interaction modulate ankyrin binding to CD44. In the present study, we showed that protein 4.1 and the recombinant 30-kDa domain of protein 4.1 (r30kDa)\textsuperscript{1} bind to the cytoplasmic domain of CD44. We also documented that Ca\textsuperscript{2+} and calmodulin re-
Modulation of CD44-Ankyrin Interaction by Protein 4.1

Binding Assay by Resonant Mirror Detection—Protein-protein interactions and protein-peptide interactions were studied using the resonant mirror detection method (30–32) of the IAsys™ system (Affinity Sensors). Recombinant CD44cyt and the synthetic peptides were immobilized on the surface of aminosilane- and BSA-coated cuvettes, respectively. The rCD44cyt was immobilized on the aminosilane-coated cuvette according to the manufacturer’s instructions with slight modifications. All experimental procedures were carried out at 25°C with constant stirring. In brief, an aminosilane-coated cuvette was washed with 0.2 ml of distilled water followed by activation with 0.2 ml of 2 mM bis(sulfosuccinimidyl) suberate. 0.1 ml of CD44cyt in 0.1 mg/ml PBS (phosphate-buffered saline; 10 mM Na₂HPO₄/NaH₂PO₄, pH 7.4, containing 0.15 mM NaCl) was added to the cuvette and incubated for 30 min at 25°C. After washing with PBS, 2 mg/ml BSA was added to the cuvette to reduce nonspecific binding. The synthetic peptides were immobilized onto the cuvette surface through BSA. In brief, 0.1 ml of BSA (2 mg/ml in PBS) was added to the bis(sulfosuccinimidyl) suberate-activated aminosilane-coated cuvette and incubated for 20 min. After washing with PBS, the cuvette was rinsed three times with 20 ml PBS. After PBS washings, surface-bound BSA was activated with 200 mM EDAC and 58 mM N-hydroxysuccinimide for 10 min. Synthetic peptides in PBS (1 mg/ml) were reacted with activated BSA by incubation for 30 min at 25°C. After the reaction, the cuvettes were washed with PBS, and the unoccupied sites on the surface were blocked with 1% ethanolamine. Cuvettes with immobilized glutathione S-transferase alone or BSA alone were prepared to serve as negative controls for the binding studies. All the binding assays were carried out in 20 mM imidazole HCl, pH 7.2, buffer containing 0.15 mM NaCl except for those assessing calmodulin effects on binding. To determine calmodulin effects, r30kDa was preincubated with 2 μM calmodulin in 20 mM imidazole HCl, pH 7.2, buffer containing 0.1 mM NaCl (buffer A) and either 0.1 mM EGTA or 1.1 mM CaCl₂ and 1.0 mM EDTA at 25°C for 30 min. The same buffers were also used during the binding assay to evaluate calmodulin effects. The binding of protein “B” from solution to protein “A” immobilized on the cuvette surface was monitored by measuring resonant mirror response in arc seconds in the IAsys™ system. The time-dependent changes in arc seconds provided information concerning real-time changes in surface concentration of protein complexes formed on the sensor surface (30–32). The resulting binding curve (arc seconds versus time) was analyzed using the software package FASTfit™ (Affinity Sensors). The rate of formation of the protein complex [AB] on the surface at different concentrations of protein B added to the cuvette is given by

\[
dR/dt = k_B[B]R_{\text{max}} - (k_1[B] + k_2)R, \quad (\text{Eq. 1})
\]

where [B] is the concentration of protein B used in deriving the binding curve, \(R_t\) is the response in arc seconds at time \(t\), \(R_{\text{max}}\) is the maximum value of \(R\) at saturating concentrations of protein B, and \(k_1\) and \(k_2\) are association and dissociation rate constants, respectively. Furthermore, the association of protein B with immobilized protein A can be described by the following pseudo-first order equation,

\[
R_t = R_0 + R_{\text{max}} (1 - e^{-k_{\text{on}}t}) \quad (\text{Eq. 2})
\]

where \(R_0\) is the initial response and \(k_{\text{on}}\) is given by Equation 3.

\[
k_{\text{on}} = k_1[B] + k_2 \quad (\text{Eq. 3})
\]

Using the above described relationships, the FASTfit™ program uses an iterative curve fitting procedure to derive values for \(k_{\text{on}}\) as a function of [B] which best fit the arc seconds versus time binding data recorded at varying concentrations of protein B. Based on the relationship outlined in Equation 3, the slope of the plot of \(k_{\text{on}}\) versus [B] provides the value for \(k_1\) and the intercept provides the value for \(k_2\).
(32). The dissociation constant from this form of kinetic analysis (termed \( K_{D,kin} \)) is then calculated as shown in Equation 4.

\[
K_{D,kin} = k_f/k_a
\]

(Dissociation rate constants can also be derived by measuring the loss of signal (arc seconds) as a function of time immediately after the replacement of the binding buffer containing protein B with a protein-free solution. The FASTFIT\textsuperscript{TM} program is then used to derive the value of \( k_a \) by deriving the best fit of the dissociation curve data to the relationship

\[
R_t = R_{eq} \times e^{-k_d t}
\]

(Eq. 5)

(32). For self-consistency, the values of \( k_a \) derived from these two different approaches must be very similar, and this criterion was met by all of the binding studies reported here.

Dissociation constant by Scatchard analysis (termed \( K_{D,Scat} \)) were also derived from the binding data. The maximal extent of binding (\( R_{eq} \)) at various concentrations of [B] was derived from binding curves. Because

\[
K_{D,Scat} = 1/K_a
\]

(Eq. 7)

In the present study the \( K_{D,Scat} \) derived under a variety of experimental conditions closely matched the corresponding \( K_{D,kin} \) values calculated.

At least two cuvettes were used to determine various binding constants, and the derived values differed by less than 10% between the two measurements. The cuvettes were reused after cleaning with HCl. Original binding curves could be replicated after HCl washes, implying that the washing procedure used did not denature the bound ligands.

**Immunofluorescence Studies—** Human epidermal keratinocytes at passage 2 were grown on coverslips in KGM medium containing 0.15 M NaCl) was introduced into the aminosilane cuvette with an ionic strength was determined using the IAsys\textsuperscript{TM} system. After this washing step, the response signal decreased and reached a new lower plateau value. The cuvette was subsequently washed with 20 mM HCl to remove bound proteins (HCl).

**FIG. 2. Typical tracings from the IAsys\textsuperscript{TM} system to monitor interaction between rCD44cyt with protein 4.1 (panel A) and with r30kDa (panel B), 200 \( \mu \)l of protein 4.1 (110 \( \mu \)g/ml) and r30kDa (98 \( \mu \)g/ml) in the binding buffer (20 mM imidazole HCl, pH 7.2, containing 0.15 M NaCl) was introduced into the aminosilane cuvette and immobilized rCD44cyt. The binding response as monitored by an increase in arc seconds reached a plateau value at 3.5 min. After the equilibrium state was reached, the cuvette was washed with protein-free binding buffer (washing). After this washing step, the response signal decreased and reached a new lower plateau value. The cuvette was subsequently washed with 20 mM HCl to remove bound proteins (HCl).**

**RESULTS**

**Binding of Protein 4.1 via Its 30-kDa Domain to CD44**—The ability of the cytoplasmic domain of CD44 (CD44cyt) and various synthetic peptides from the cytoplasmic domain (Fig. 1) to bind to either protein 4.1 or its 30-kDa domain at physiologic ionic strength was determined using the IAsys\textsuperscript{TM} system. After the addition of protein 4.1 to rCD44cyt immobilized on the cuvette, the binding response (detected by the resonant mirror detection system in arc seconds) increased, reaching a plateau after 3.5 min (Fig. 2A). After washing the cuvette with protein-free buffer, the binding response signal decreased to a new plateau value that was significantly higher than baseline, implying that a specific interaction had occurred between protein 4.1 and rCD44cyt. A similar binding response was observed after the addition of either the native or recombinant 30-kDa domain of protein 4.1 to rCD44cyt (Fig. 2B). In contrast, heat-denatured r30kDa (5 min at 100 °C) did not bind to CD44 (data not shown). The other functional domains of protein 4.1 (16-, 10-, and 22-24-kDa domains) also did not bind to CD44 (data not shown). Furthermore, protein 4.1 and r30kDa did not bind to either glutathione S-transferase or BSA (data not shown). These data imply that protein 4.1 binds to CD44 through its 30-kDa domain. Analysis of the binding response curves obtained at varying concentrations of protein 4.1 and r30kDa provided kinetic rate constants for association and dissociation of these proteins from CD44 (Table I). Protein 4.1 and r30kDa bound to the cytoplasmic domain of CD44 with \( K_{D,kin} \) and \( K_{D,Scat} \) values on the order of \( 10^{-7} \) M (Table I).

**Protein 4.1 Binding Domain of CD44—** Binding of the 30-kDa domain of protein 4.1 to various synthetic peptides from the cytoplasmic domain of CD44 (Fig. 1) was quantitated to define the protein 4.1 binding domain of CD44. r30kDa bound to immobilized peptides CD44-(1-2) and CD44-1 from the cytoplasmic domain of CD44. \( K_{D,kin} \) and \( K_{D,Scat} \) values were once again on the order of \( 10^{-7} \) M (Table I). However, no binding occurred between the 30-kDa domain of protein 4.1 and synthetic peptides CD44-2, CD44-3, CD44-4, and CD44-5 (Table I). These findings, along with the observation that the synthetic peptide CD44-1 binds to protein 4.1 with a binding affinity similar to that of the entire cytoplasmic domain, imply that the sequence motif NSRPRCQKKKLVI is critical for binding of CD44 to protein 4.1. To define the minimal peptide sequence involved in CD44 binding to protein 4.1, the binding capacity of four overlapping peptides spanning the CD44-1 sequence was determined. Each overlapping peptide was composed of seven amino acids. r30kDa bound to peptides CD44-1a (N\textsuperscript{628}SRRRCG) and CD44-1c (Q\textsuperscript{635}KKKLVI) with a \( K_{D,kin} \) of 2.75 \( \times 10^{-7} \) M (\( k_a = 2.69 \times 10^{7} \) M\(^{-1}\) s\(^{-1}\), \( k_d = 0.74 \times 10^{-2} \) s\(^{-1}\)), and 3.03 \( \times 10^{-7} \) M (\( k_a = 3.04 \times 10^{7} \) M\(^{-1}\) s\(^{-1}\), \( k_d = 0.92 \times 10^{-2} \) s\(^{-1}\)), respectively. These values were similar to the value obtained with the entire cytoplasmic domain of CD44 (Table I). On the other hand, r30kDa bound to peptides CD44-1b (R\textsuperscript{631}RCQKKK) and CD44-1d (K\textsuperscript{637}KLVINS) with much lower affinities, \( K_{D,kin} \) values of 1.03 \( \times 10^{-6} \) M and 5.28 \( \times 10^{-6} \) M, respectively. Taken together these data imply that the basic amino acid clusters RRR and KKK in the cytoplasmic domain of CD44 are involved in high affinity interactions with protein 4.1.

**Colocalization of CD44 and Protein 4.1 in Keratinocytes**—To document potential interaction between CD44 and protein 4.1 in native cell membranes, the relative localization of both these protein components in keratinocyte membranes was determined by immunofluorescence microscopy using antibodies specific for CD44 and protein 4.1. Epitopes for protein 4.1 (green), detected using two different antibodies, were found in...
the interior of the cell as well as at the peripheral cell membrane (Fig. 3). CD44 epitopes (red) localized exclusively at the plasma membrane (Fig. 3). The yellow coloration produced in areas where red and green signals coincide was a prominent feature of the plasma membrane, indicating colocalization of CD44 and protein 4.1 in the plasma membrane of keratinocytes. Samples with nonimmune IgG (Fig. 3) or with primary antibody omitted showed no fluorescent patterns.

**Regulation of CD44-Protein 4.1 Interaction by Ca\(^{2+}\) and Calmodulin**—To determine if CD44-protein 4.1 interaction can be regulated by Ca\(^{2+}\) and calmodulin, we quantitated the binding of the synthetic peptide CD44-(1+2) to r30kDa in the presence and absence of Ca\(^{2+}\) and calmodulin (Table II). Either 2 μM calmodulin or 100 μM Ca\(^{2+}\) alone had no effect on either the association or dissociation rate constants of interaction between r30kDa and CD44-(1+2) (Table II). However, in the presence of both Ca\(^{2+}\) and calmodulin, there was a 16-fold increase in the dissociation rate constant (\(k_d\)), with little or no effect on the association rate constant (\(k_a\)) (Table II). These changes were reflected by a 16-fold increase in the \(K_{D,\text{kin}}\) value for the interaction between r30kDa and CD44-(1+2) in the presence of both Ca\(^{2+}\) and calmodulin. Similar effects of Ca\(^{2+}\) and calmodulin were seen when intact protein 4.1 was used in the binding studies (data not shown). These data imply that Ca\(^{2+}\)-calmodulin binding to protein 4.1 can accelerate the dissociation of protein 4.1 from CD44.

The Ca\(^{2+}\) concentration dependence of calmodulin-induced protein 4.1 dissociation from CD44 was quantitated (Fig. 4). At Ca\(^{2+}\) concentrations greater than 0.1 μM (pCa < 7), the extent of r30kDa binding to CD44-(1+2) started to decline, and maximal inhibition of binding was noted at Ca\(^{2+}\) concentrations of 100 μM and higher (pCa < 4). A half-maximal effect was seen at a Ca\(^{2+}\) concentration of 7 μM. Interestingly, calmodulin inhibitors W-7 and W-13, at concentrations up to 100 μM, did not inhibit Ca\(^{2+}\) and calmodulin-induced dissociation of CD44 from protein 4.1 (data not shown).

**Binding of Ankyrin-R to CD44**—To define the ankyrin-binding domain of CD44, the ability of the CD44 cytoplasmic domain and various synthetic peptides from the cytoplasmic domain (Fig. 1) to bind ankyrin was quantitated (Fig. 5A). Ankyrin-R bound to the cytoplasmic domain of CD44 and peptides CD44-(1+2) and CD44-2 with \(K_{D,\text{kin}}\) and \(K_{D,\text{Scat}}\) Values on the order of \(10^{-7}\) M (Table III). However, no binding was seen between ankyrin-R and synthetic peptides CD44-1, CD44-3, CD44-4, and CD44-5 (Table III). Thus, protein 4.1 and ankyrin-R bind to distinctly different sequence motifs in the cytoplasmic domain of CD44, with protein 4.1 binding to CD44-1 (NISGRPRRRCGKCLT) and ankyrin-R binding to CD44-2 (NISGRPRRRCGKCLT).

**Regulation of Ankyrin Binding to CD44 by Protein 4.1**—To determine whether protein 4.1 can regulate ankyrin-CD44 interaction, r30kDa and ankyrin-R were added sequentially to CD44-(1+2) immobilized on the aminosilane cuvette. After the addition of r30kDa, the binding response curve showed a specific interaction between r30kDa and CD44-(1+2) (Fig. 5B). Subsequent addition of ankyrin-R to the cuvette failed to produce a signal that corresponded to specific ankyrin binding (Fig. 5B). Thus, although ankyrin-R can bind to CD44-(1+2) in the absence of r30kDa (Fig. 5A), it cannot bind to CD44-(1+2) prebound to r30kDa (Fig. 5B). Interestingly, addition of 100 μM Ca\(^{2+}\) and 10 μM calmodulin in buffer A to the r30kDa-CD44 complex before the addition of ankyrin-R partially restored (to approximately 70% of normal) the ability of ankyrin to bind to CD44. These data suggest that protein 4.1 can modulate the interaction of ankyrin with the cytoplasmic domain of CD44. Of note, ankyrin-R did not bind calmodulin either in the absence
Modulation of CD44-Ankyrin Interaction by Protein 4.1

TABLE II
Effect of Calmodulin on r30kDa binding to CD44-(1+2)

| Condition | Calmodulin | $K_{d}$ | $K_{D,kin}$ | $K_{D,stat}$ |
|-----------|------------|---------|-------------|-------------|
| EGTA      | –          | 5.62 ± 0.04 × 10⁴ | 1.61 ± 0.05 × 10⁻⁷ | 2.86 × 10⁻⁷ |
|           | +          | 5.03 ± 0.05 × 10⁴ | 1.74 ± 0.05 × 10⁻⁷ | 3.46 × 10⁻⁷ |
| Ca²⁺      | –          | 6.62 ± 0.04 × 10⁴ | 1.50 ± 0.02 × 10⁻⁷ | 2.40 × 10⁻⁷ |
|           | +          | 5.13 ± 0.02 × 10⁴ | 2.03 ± 0.01 × 10⁻⁷ | 3.96 × 10⁻⁷ |

or presence of 100 μM Ca²⁺ (data not shown). Furthermore, although Ca²⁺ and calmodulin accelerated the dissociation of protein 4.1 from CD44, they had no effect on the CD44-ankyrin-R interaction (data not shown).

DISCUSSION

The present study documents that protein 4.1 binds to the cytoplasmic domain of CD44 through its amino-terminal 30-kDa domain. Immunolocalization studies documenting colocalization of protein 4.1 and CD44 in the plasma membrane of keratinocytes further suggest that the CD44-protein 4.1 interaction is likely to be physiologically relevant in the intact cell.

Using segments of synthetic peptides spanning the entire cytoplasmic domain of CD44, the protein 4.1 binding domain of CD44 was mapped to the sequence motif NSRRRCGQKKLVI. Further studies with smaller CD44 peptide segments showed that SRRRC and QKKKL sequence motifs in CD44 bind to protein 4.1 with affinities similar to that of the entire cytoplasmic domain. Interestingly, Jons and Drechselkahn (9) showed previously that arginine-rich clusters of the cytoplasmic domain of band 3, LRRRY or IRRRY, serve as the major binding sites for protein 4.1 in human erythrocytes.

Ca²⁺-independent binding of calmodulin to the 30-kDa domain of protein 4.1 has been reported previously (33). Lombardo and Low (12) showed that Ca²⁺ and calmodulin can decrease the affinity of interactions between band 3 and glycoporphin C with protein 4.1. In an analogous fashion, the present study demonstrates that the CD44-protein 4.1 interaction is similarly modulated by Ca²⁺ and calmodulin. These findings raise the possibility that increases in the intracellular concentration of calcium can modulate binding of protein 4.1 to CD44 as well as to its other binding partners.

The ankyrin family of proteins links various membrane proteins to the actin cytoskeleton through interaction with spectrin or fodrin (6, 34–36). For example, in erythrocytes, ankyrin-R links band 3 to β-spectrin. In non-erythroid cells, ankyrin(s) binds to various membrane proteins including Na⁺/K⁺ ATPase (37, 38) voltage-dependent Na⁺ channels (39), amiloride-sensitive Na⁺ channels (40), inositol 1,4,5-trisphosphate receptor (41), and CD44 (42, 43). In the present study, we confirmed the binding of ankyrin-R to CD44 and have mapped the ankyrin binding domain of CD44 to the sequence motif NSNGAVEDRKPSSL. Thus we have documented that both ankyrin-R and protein 4.1 bind to distinctly different regions of the cytoplasmic domain of CD44.

Comparison of our findings with current understanding of ankyrin and protein 4.1 interactions in the erythrocyte membrane suggests some similarities between band 3 and CD44 interactions with the membrane skeleton. For example, cytoplasmic domains of both band 3 and CD44 interact with ankyrin-R and protein 4.1. In addition, Ca²⁺ and calmodulin modulate the interaction of protein 4.1 with both band 3 and CD44. We have shown previously that protein 4.1 binding to band 3 modulates its interaction with ankyrin-R. Specifically, protein 4.1 binding to band 3 resulted in a marked reduction in the high affinity binding sites for ankyrin on band 3, and this in turn resulted in marked alterations in membrane mechanical function (13). By analogy, we propose that the present finding of protein 4.1 regulating ankyrin binding to CD44 may also modulate membrane function.

Based on our findings, we propose the following speculative model for a potential role for ankyrin-CD44-protein 4.1 interactions in cells expressing CD44 (Fig. 6). Calmodulin is normally present in the cytosol of cells at μM concentrations, and the intracellular Ca²⁺ concentration is maintained at 1 μM or less. Under these conditions, protein 4.1 is bound to the cytoplasmic domain of CD44 with high affinity, and as a result, the ankyrin-CD44 interaction is inhibited (Fig. 6). When the cytosolic Ca²⁺ concentration exceeds 1 μM, the Ca²⁺-calmodulin complex reduces the affinity of protein 4.1-CD44 interaction, and this in turn leads to ankyrin binding to CD44 (Fig. 6). Ankyrin binding to the cytoplasmic domain of CD44 could induce a conformational change in CD44 enabling binding of ligands to the extracellular domain of CD44, such as hyaluronic acid (HA). Although no direct evidence is currently available to support a conformational change in CD44 after ankyrin binding, the plausibility of our proposed model is supported by...

![Fig. 4. Ca²⁺ concentration dependence of protein 4.1 binding to CD44. r30kDa binding to CD44-(1+2) was measured at various concentrations of Ca²⁺ in the presence of 5 μM calmodulin. Ca²⁺ concentrations were maintained by a calcium-EGTA buffer system. The maximal extent of binding under different experimental conditions was quantitated as described under “Experimental Procedures.” Maximal binding in the presence of EGTA was used to normalize binding in the presence of varying concentrations of Ca²⁺. pCa represents ionized Ca²⁺ concentration. With increasing Ca²⁺ concentrations, the amount of r30kDa binding to CD44-(1+2) decreased.](image-url)
several interesting findings. It has been reported that CD44 mediates interactions of keratinocytes and various other types of cells with extracellular matrices such as HA and collagen (44) and that elevation of cytosolic concentrations of Ca$^{2+}$ stimulates HA-induced receptor (CD44) capping and adhesion of cells to HA-coated plates (45–48).

Immunochemical analysis has demonstrated the presence of protein 4.1 superfamily members such as ezrin, radixin, and moesin (designated ERM family) in keratinocytes and other cell types. Homology between the amino-terminal domain of the ERM family of proteins and the 30-kDa domain of protein 4.1 was demonstrated (2). W. Nunomura, Y. Takakuwa, R. Tokimitsu, S. W. Krauss, M. Kawanashima, and N. Mohandas, unpublished data.

**TABLE III**

| Peptides   | $k_a$ | $k_d$ | $K_{D_{\text{bind}}}$ | $K_{D_{\text{Scat}}}$ |
|------------|-------|-------|-----------------------|-----------------------|
| rCD44cyt   |       |       |                       |                       |
| CD44-(1+2) |       |       |                       |                       |
| CD44-1     | No binding | No binding | No binding | No binding |
| CD44-2     | No binding | No binding | 1.11 $\times 10^{-7}$ | 4.21 $\times 10^{-7}$ |
| CD44-3     | No binding | No binding | No binding | No binding |
| CD44-4     | No binding | No binding | No binding | No binding |
| CD44-5     | No binding | No binding | No binding | No binding |

**Fig. 5. Typical tracings from the IAsys™ system to monitor interaction between ankyrin-R and CD44-(1+2).** Panel A, to document ankyrin binding to CD44, 100 µl of binding buffer containing ankyrin-R (80 µg/ml) was introduced into the aminosilane cuvette with immobilized CD44-(1+2). After the binding response of ankyrin reached a plateau value, the cuvette was washed with protein-free binding buffer (washing). Specific binding of ankyrin is revealed by a sustained net increase in response signal after washing with binding buffer. Panel B, to document the effect of protein 4.1 on ankyrin-R binding to CD44, 100 µl of binding buffer containing r30kDa (63 µg/ml) was first introduced into the aminosilane cuvette with immobilized CD44-(1+2). After the binding response of r30kDa reached a plateau value, the cuvette was washed with the binding buffer (washing), and 100 µl of binding buffer containing ankyrin-R (80 µg/ml) was subsequently introduced into the cuvette. The absence of a sustained net change in response signal following washing with the binding buffer implies absence of specific ankyrin-R binding under these experimental conditions. The washing of the cuvette with 20 mM HCl removed bound proteins (HCl).

**Fig. 6. Proposed model for CD44-ankyrin interactions.** Calmodulin (CaM) is normally present in the cytosol of cells at µM concentrations, and the intracellular Ca$^{2+}$ concentration is maintained at 1 µM or less. Under normal conditions with low intracellular Ca$^{2+}$ concentration, protein 4.1 is bound to the cytoplasmic domain of CD44 with high affinity, and as a result ankyrin cannot bind to CD44. However, with increasing cytosolic Ca$^{2+}$ concentration, the Ca$^{2+}$-calmodulin complex reduces the affinity of protein 4.1-CD44 interaction, and this in turn leads to ankyrin binding to CD44. Ankyrin binding could lead to a conformational change in the CD44 with subsequent binding of ligands to the extracellular domain of CD44 such as HA.
4.1 involved in binding to band 3, glycoporphin C, and CD44 is approximately 36% (49, 50). Furthermore, members of ERM family of proteins are known to bind to various membrane proteins, including CD44 (51). However, the sequence motif LEEDY in the 30-kDa membrane binding domain of protein 4.1 responsible for binding of protein 4.1 to band 3 (9) is absent in the ERM family of proteins (52–54). Thus the binding motifs of ERM proteins responsible for interaction with integral membrane proteins appear to differ from that of protein 4.1.

At present the relative contributions of protein 4.1 and the ERM family of proteins to interaction with various membrane proteins remains to be defined.

Previous studies provided significant insights into the important contribution of protein 4.1 in regulating erythrocyte membrane function. The results from the present study raise the possibility that protein 4.1, which is expressed widely in various cell types including epithelial cells and fibroblasts, may regulate the organization and function of other ankyrin-linked integral membrane proteins in these cells.

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