The ANK Repeats of Erythrocyte Ankyrin Form Two Distinct but Cooperative Binding Sites for the Erythrocyte Anion Exchanger*

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The 24 ANK repeats of the membrane-binding domain of ankyrin form four folded subdomains of six ANK repeats each. These four repeat subdomains mediate interactions with at least seven different families of membrane proteins. In the erythrocyte, the main membrane target of ankyrin is the Cl⁻/HCO₃⁻ anion exchanger. This report presents the first evidence that ankyrin contains two separate binding sites for anion exchanger dimers. One site utilizes repeat subdomain two (repeats 7-12) while the other requires both repeat subdomains three and four (repeats 13-24). The two sites are positively coupled with a Hill coefficient of 1.4. Since the anion exchanger exists as a dimer in the membrane, the presence of two binding sites on ankyrin allows ankyrin to interact with four anion exchangers simultaneously. These findings provide a direct demonstration of the versatility of ANK repeats in protein recognition, and have important implications for the organization of ankyrin-linked integral membrane proteins in erythrocytes as well as other cells.

The spectrin-based membrane skeleton is an interlocking network of proteins which underlies the plasma membrane. The skeleton was first identified in erythrocytes, but it is also present under specialized regions of plasma membrane of cells in many tissues. The membrane skeleton is comprised of heterotetramers of α- and β-spectrin which form multiple, long range cross-links between cortical actin filaments. This structure is then linked to the membrane primarily by ankyrin, which possesses binding sites for β-spectrin and at least seven membrane proteins (1-2).

In human erythrocytes, the interaction between ankyrin and the cytoplasmic domain of the Cl⁻/HCO₃⁻ anion exchanger (Band 3) provides a major linkage between the spectrin skeleton and the plasma membrane (3-5). This linkage gives the erythrocyte membrane elastic properties which allows the plasma membrane to deform without vesiculation. Mutations or reductions in ankyrin which disrupt the linkage with the anion exchanger decouple the structural support of the spectrin skeleton from the membrane and result in severe hemolytic spherocytosis and anemia (2, 6, 7).

The ankyrin binding activity has been localized to the N-terminal, 89-kDa domain of ankyrin (8). The amino acid sequence of the 89-kDa domain is dominated by a tandem array of 24 ANK repeats each. These four repeat subdomains mediate interactions with at least seven different families of membrane proteins. In the erythrocyte, the main membrane target of ankyrin is the Cl⁻/HCO₃⁻ anion exchanger. This report presents the first evidence that ankyrin contains two separate binding sites for anion exchanger dimers. One site utilizes repeat subdomain two (repeats 7-12) while the other requires both repeat subdomains three and four (repeats 13-24). The two sites are positively coupled with a Hill coefficient of 1.4. Since the anion exchanger exists as a dimer in the membrane, the presence of two binding sites on ankyrin allows ankyrin to interact with four anion exchangers simultaneously. These findings provide a direct demonstration of the versatility of ANK repeats in protein recognition, and have important implications for the organization of ankyrin-linked integral membrane proteins in erythrocytes as well as other cells.

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**EXPERIMENTAL PROCEDURES**

**Materials**

¹²⁵I-Labeled Bolton-Hunter was obtained from ICN Radiochemicals. All chromatography matrices were obtained from Pharmacia Biotech Inc. Neutavidin and NHS-LC-biotin were purchased from Pierce Chemical Co.; DNase I and bovine serum albumin, from U. S. Biochemical Corp.; and Taq polymerase, from Perkin-Elmer Cetus. Isopropyl-thiogalactopyranoside was obtained from ICN Biochemicals. The pGEMEX expression vector was from Promega. Triton X-100 was from Boehringer Mannheim. The beads used were 0.4-µm size selected proplyoxy-derivatized, non-porous latex beads which were custom synthesized by Bangs, Inc. All other chemicals were obtained from Sigma.

**Methods**

**Bacterial Expression and Purification**—Protein expression of recombinant protein constructs was performed using an isopropyl-1-thio-β-D-galactopyranoside inducible T7 expression system with the pGEMEX (Promega) vector (12, 13). Expressed protein products were purified from bacterial lysates using gel filtration and ion exchange chromatography (see Fig. 1).

**Binding Assays**—Membrane binding assays were performed as described (8). Reagents for the bead assays were produced using the following procedures. The cytoplasmic domain of the anion exchanger (CD8) was purified and labeled with ¹²⁵I-Bolton-Hunter reagent as
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Fig. 1. Purification and map of repeat domain constructs. Recombinant protein constructs were designed, based upon the repeat subdomains present in the 89-kDa domain membrane-binding domain of ankyrin (upper panel). Proteins containing one (D1, D2, D3, and D4) and two (D1-D2, D2-D3, and D3-D4) repeat subdomains and native 89-kDa domain containing all four repeat subdomains were uniformly loaded on an SDS-polyacrylamide gel with 1.5 µg of protein/lane. The gel (lower left) was electrophoresed and stained with Coomassie Blue. The name of the protein is used as the designation for each lane. The region contained within each construct is shown in the lower right. The numbers refer to the repeats. N designates the first 9-10 amino acids from the N terminus of ankyrin to the start of the first repeat. H refers to the “hinge region,” a 32-amino acid stretch from the end of the last repeat to the chymotryptic site separating the membrane-binding domain from the spectrin-binding domain. The residues contained within each protein are as follows: D1, 1-204; D2, 205-402; D3, 403-600; D4, 601-827; D1-D2, 1-402; D2-D3, 205-600; D3-D4, 403-827; and the 89-kDa domain, 1-827.

Fig. 2. Hill plot analysis of the interaction of native 89-kDa domain and construct D3-D4 with stripped erythrocyte membranes. Membrane binding assays were performed using 126I-labeled 89-kDa domain (220,000 cpm/pmol) and D3-D4 (33,100 cpm/pmol) with KI stripped inside-out erythrocyte membranes (see “Experimental Procedures”). Affinities were calculated from the zero intercept on the y axis. Construct D3-D4 displayed a linear plot with a $K_d$ of 10 nM and a Hill coefficient of 0.95. The 89-kDa domain interaction had a $K_d$ of 1.5 nM, but had a biphasic Hill plot with a Hill coefficient of 2.2 at concentrations below the $K_d$ and a Hill coefficient of 0.9 above the $K_d$. In the Hill plot, [free ligand] is the concentration of free 89-kDa domain or D3-D4 in solution at each point. $\theta$ is the fraction of sites on the membrane occupied by labeled protein. C, 89-kDa domain; D, D3-D4.

Fig. 3. CDB3 interaction with native ankyrin. The 216-kDa isoform of ankyrin (band 2.1) was purified from erythrocytes and coupled to latex beads. These beads were assayed for activity as described under “Experimental Procedures.” The saturation plot of $^{125}$I-labeled CDB3 (6932 cpm/pmol) with the ankyrin coupled beads is displayed in panel A. Concentration (nm) is the concentration of CDB3 polypeptide. Bound (fmol) is the femtomoles of CDB3 bound to the latex beads. Panel B contains the corresponding Scatchard plot and shows a partially concave interaction. Bound (fmol) is again the femtomoles of CDB3 bound. Bound/Free (fmol/nM) is the ratio between the femtomoles of CDB3 bound versus the nanomolar concentration of CDB3 free in solution. The Hill plot in panel C indicates that the interaction with native ankyrin has positive cooperativity with a Hill coefficient of 1.3.

described (14). The beads were prepared as follows: 0.4-µm epoxy-activated latex beads (Bangs) were reacted with Neutravidin (Pierce) with end-over-end mixing for 72 h at 4 °C in coupling buffer containing 50 mM sodium phosphate, pH 8.0, 1 mM NaCl, and 1 mM NaN₃. Unreacted sites were quenched by adding Tris to 100 mM, pH 8.0, and incubating overnight. Non-specific protein sites were blocked by adding bovine serum albumin to 0.5 mg/ml for 2 h. Beads were then washed three times with coupling buffer. ANK-repeat constructs were biotinylated using the Pierce procedure with NHS-LC-biotin at 4 °C. After removing unreacted biotin by dialysis into coupling buffer plus 1 mM dithiothreitol, biotinylated constructs were added to the beads and reacted overnight at 4 °C. Unbound protein was removed by washing three times with buffer containing 10 mM sodium phosphate, pH 7.2, 1 mM NaCl, 1 mM NaN₃, 1 mM dithiothreitol, and 0.05% Tween 20. The beads were then suspended to 2.5% (w/v) in buffer containing 10 mM sodium phosphate, pH 7.2, 100 mM NaCl, 1 mM NaN₃, 1 mM dithiothreitol, and 0.1% Tween 20.

The ability of labeled anion exchanger cytoplasmic domain (CDB3) to interact with the various ankyrin-conjugated beads was assessed as follows. 0.8% (w/v) ANK repeat-conjugated beads were incubated with increasing concentrations of $^{125}$I-labeled CDB3 in a buffer containing 10
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**RESULTS**

Comparison of the 89-kDa Domain and Repeat Construct D3-D4 Interaction with Ankyrin-depleted Inside-out Erythrocyte Vesicles — The 89-kDa domain is 30–40% more active than a construct containing just repeat domains three and four (construct D3-D4; see Fig. 1) in competing for binding of ankyrin to the anion exchanger in erythrocyte membranes (8). In order to better understand the basis for higher activity of the 89-kDa domain, native 89-kDa domain and construct D3-D4 expressed in bacteria (Fig. 1) were labeled with $^{125}$I and assayed for their ability to interact with ankyrin-depleted inside-out erythrocyte vesicles (Fig. 2). A Hill plot of the binding data revealed half-maximal binding of D3-D4 at 10 nM with a Hill coefficient of 0.95. In contrast, the Hill plot for the 89-kDa domain demonstrated half-maximal binding at 1 nM with a Hill coefficient of 0.9. The enhanced cooperativity at low occupancy explains the stronger apparent affinity of the 89-kDa domain for anion exchanger sites in erythrocyte membranes. This difference in activity implies that N-terminal subdomains one and/or two participate in the association of the 89-kDa domain with the anion exchanger.

The Anion Exchanger Cytoplasmic Domain Exhibits Positive Cooperativity in the Interactions with Immobilized Ankyrin and
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The affinity of $^{125}$I-labeled CDB3 for D1-D2 and D2-D3 coupled beads was assessed as described under "Experimental Procedures." Panel A shows a saturation plot of the interaction of labeled CDB3 (5136 cpm/pmol) with immobilized D1-D2. Panel B contains the saturation plot of labeled CDB3 with immobilized D2-D3. Interactions with both D1-D2 and D2-D3 displayed biphasic Scatchard plots (panels C and D, respectively). A $K_d$ of 38 nM for the D1-D2 beads was calculated from the high affinity interaction seen in the Scatchard plot in panel C. A $K_d$ of 10 nM for construct D2-D3 was calculated from the Scatchard plot in panel D.

the 89-kDa Domain — The ability of the cytoplasmic domain of the anion exchanger (CDB3) to interact with whole ankyrin, the 89-kDa domain, and various repeat subdomains was examined by immobilizing these proteins to 0.4-µm latex beads (see "Methods") and measuring the ability of $^{125}$I-labeled CDB3 to interact with the derivatized beads. Assaying interactions in this manner has the advantage that affinities are measured using saturating concentrations of the anion exchanger with respect to a fixed amount of ankyrin. By saturating the anion exchanger, the bead assay fills anion exchanger sites on ankyrin and allows all potential sites for the anion exchanger to be identified. In contrast, membrane binding assays (Fig. 2) measure affinities using saturating concentrations of the ankyrin regions relative to a fixed number of anion exchangers in erythrocyte membranes. The membrane assays therefore only allow detection of the highest affinity site for the anion exchanger on ankyrin.

Binding of CDB3 to immobilized 89-kDa domain (Fig. 3) was cooperative as evidenced by a concave Scatchard plot (Fig. 3B) and a Hill coefficient of 1.4 (Fig. 3C). Half-maximal binding occurred at 20 nM based on the Hill plot. The interaction of CDB3 with immobilized native ankyrin (protein 2.1) (Fig. 4) was similar to the interaction with immobilized 89-kDa domain. As with the 89-kDa domain, the interaction with whole ankyrin displayed a slightly sigmoidal saturation curve. The Scatchard plot was concave indicating positive cooperativity. The Hill plot quantified the cooperativity with a Hill coefficient of 1.3. The calculated $K_d$ of 40 nM for the interaction of CDB3 with ankyrin was only 2-fold weaker than that of CDB3 with the 89-kDa domain. The overall similarity between the two interactions support the conclusion that the 89-kDa domain contains all of the activity of ankyrin for the interaction with the anion exchanger.

Construct D3-D4 exhibited half-maximal binding at 40 nM, but did not display the positively cooperative interaction of the 89-kDa domain (Fig. 5) (Hill plot not shown). The issue of whether the binding site present on construct D3-D4 required both subdomains three and four was addressed by evaluating binding activity of the individual subdomains (Fig. 5). Neither subdomains three nor four were active alone, indicating that both subdomains are required for the interaction with the anion exchanger (Fig. 5).

The positive cooperativity under the assay conditions of Figs. 3 and 4 implies that ankyrin and the 89-kDa domain contain multiple binding sites for anion exchanger dimers. Since construct D3-D4 lacks cooperativity, the N-terminal subdomains of ankyrin provide either a second site for the anion exchanger or are indirectly responsible for the positive cooperativity.

Identification of a Second Site for the Anion Exchanger on the 89-kDa Domain of Ankyrin — The possibility of a second site for the anion exchanger within the first two subdomains was examined directly by measuring binding activities of a construct containing subdomains one and two (D1-D2) and a construct containing subdomains two and three (D2-D3). The D1-D2 and D2-D3 protein constructs were active and displayed biphasic Scatchard plots (Fig. 6). The D2-D3 construct had a $K_d$ of 12 nM and was more active than the D1-D2 construct which had an affinity of 38 nM.

Subdomain two is common between D1-D2 and D2-D3 constructs and this domain alone proved to be responsible for the N-terminal second binding site (see Fig. 7). Construct D2 had a $K_d$ of 12 nM and a biphasic Scatchard plot virtually identical to that of construct D2-D3. Since subdomains one and three alone had no activity and the interaction of D2 was approximately as
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**Fig. 7.** CDB3 only interacts with D2. Domains one (D1), two (D2), and three (D3) were coupled to latex beads and assayed for the ability to interact with $^{125}$I-labeled CDB3 (5136 cpm/pmol) as described under "Experimental Procedures." Panel A contains the saturation plots of these assays. $\square$, D1; $\bigcirc$, D2; $\triangle$, D3. Panel B contains the Scatchard plot for the interaction with D2 coupled beads. A 12 nM affinity was calculated from the slope of high affinity interaction in the Scatchard plot.

strong as construct D2-D3, the second site activity is likely to be completely contained in subdomain two. In this case, construct D1-D2 would be expected to have an affinity similar to that of construct D2. The lower affinity of D1-D2 may be the result of the oligomerization state of this construct. Even in 1 M NaBr, construct D1-D2 behaves as a dimer (12) and this may adversely affect the ability of construct D1-D2 to interact with the anion exchanger.

The biphasic Scatchard plots seen with constructs D2, D1-D2, and D2-D3 result either from negative cooperativity or high and low affinity sites. One possibility is that subdomain two contains a site for each polypeptide chain in the anion exchanger dimer. At moderate concentrations of dimer, both sites are used to interact with one dimer. At high concentrations of dimer, a free dimer may compete with the bound dimer for one of the sites on subdomain two. This competition may be unfavorable and thereby cause negative cooperativity. Another possibility is that there is a second site on subdomain two for the anion exchanger. This weaker interaction may be a partial site which may participate in the D3-D4 interaction and thereby contribute to the positive cooperativity seen in the complete 89-kDa domain region.

The physically distinct anion exchanger binding sites on subdomain two and on subdomains three and four were compared in terms of their sensitivity to ionic strength. The interaction between CDB3 and construct D2-D3 was completely abolished by concentrations of NaCl above 0.5 M while the interaction with D3-D4 was unaffected at concentrations as high as 1.5 M NaCl (Fig. 8). These results suggest that electrostatic interactions mediate the association with subdomain two while non-ionic contacts mediate the interaction with subdomains three and four. 1.5 M NaCl only inhibited the interaction with the native 89-kDa domain by 40-50%. These results indicate that the 89-kDa domain has two sites, one NaCl-sensitive and one insensitive. Of interest is the finding that half-maximal inhibition of binding to construct D2-D3 occurred at 0.17 M NaCl, while binding to the 89-kDa domain had a half-maximal inhibition at 0.5 M NaCl. The difference may be the result of cooperativity between the D3-D4 site and the D2 site.

**Fig. 8.** NaCl sensitivity of the interaction between CDB3 and constructs D2-D3, D34, and native 89-kDa domain. Assays were performed using $20 \text{ nM}^{125}$I-labeled CDB3 (6932 cpm/pmol) in the presence of increasing concentrations of NaCl with beads coated with constructs D2-D3 (panel A), D3-D4 (panel B), or whole 89-kDa domain (panel C). The half-maximal competition for D2-D3 bead interaction was 175 mM. The D3-D4 bead interaction could not be significantly competed with NaCl even at the highest concentrations of NaCl (1.6 M). The interaction with the 89-kDa domain coupled beads was competed 40-50% by increasing concentrations of NaCl. The half-maximal competition for the 89-kDa domain beads was 500 mM NaCl.
bridging experiment (Fig. 9), wherein CDB3 was coupled to latex beads and the ability of 125I-labeled CDB3 to interact with these beads was assessed with and without the addition of 100 nM concentration of the 89-kDa domain. Increased binding of labeled CDB3 was observed in the presence of the 89-kDa domain, with half-maximal effects at 10 nM CDB3. The difference curve was concave, presumably because at low concentrations of free CDB3, the 89-kDa domain bridges between free and bound CDB3, but at high concentrations, free CDB3 binds to both sites on the 89-kDa domain.

**DISCUSSION**

This paper presents the first evidence that ankyrin contains two separate binding sites for anion exchanger dimers. One site is located on repeat subdomain two; the other site requires both repeat subdomains three and four. The two sites can be distinguished by their different affinities and sensitivities to high ionic strength. The two sites are positively coupled with a Hill coefficient of 1.4. Since the interaction with D2 has a 4-fold higher affinity than that with the D3-D4, repeat domain two may contain the primary site of interaction with the anion exchanger. These findings provide a direct demonstration of the versatility of ANK repeats in protein recognition, and have important implications for the organization of ankyrin-linked integral membrane proteins in erythrocytes as well as other cells.

The ability of ankyrin to bind to two anion exchanger dimers helps resolve an unexplained discrepancy between the number of anion exchangers and the capacity of ankyrin-depleted erythrocyte membranes to rebind ankyrin. Each erythrocyte contains roughly 10^6 anion exchangers, assembled into 4 X 10^5 intramembrane particles comprised primarily of dimers as well as a smaller number of higher oligomers (16-18). Anion exchanger dimers can associate with ankyrin with high affinity in solution (4, 5, 19), and all anion exchangers appear to be functionally and structurally equivalent (3, 5, 19, 20). Nevertheless, ankyrin-depleted erythrocyte membranes can rebind a maximum of only 100,000–250,000 ankyrin molecules (4, 21) corresponding to a limiting stoichiometry of approximately one ankyrin for four anion exchanger polypeptides. The evidence presented in this study indicates that ankyrin contains two binding sites for anion exchanger dimers and readily explains how four anion exchangers can interact with one ankyrin molecule. The limited number of binding sites for ankyrin can be explained if anion exchanger dimers in the membrane preferentially interact with an ankyrin-anion exchanger dimer complex over ankyrin free in solution. The cooperativity of the CDB3 interaction with the 89-kDa domain and whole ankyrin (Figs. 3 and 4) provide a mechanism for this selectivity.

A higher order complex of anion exchanger with ankyrin in intact erythrocyte membranes has also been predicted by rotational diffusion studies (22). Diffusion measurements of the anion exchanger indicate that 40% of the anion exchangers (4 X 10^5 per cell) display restricted rotational diffusion (22). Ankyrin is present in approximately 10^5 copies per cell (23). Thus, assuming the immobile anion exchangers are directly coupled to ankyrin, four anion exchangers are bound to every ankyrin. The formation of this complex can be directly and simply explained by the presence of two sites on ankyrin for the anion exchanger dimer.

In the interaction with erythrocyte membranes, the 89-kDa membrane-binding domain of ankyrin exhibits positive cooperativity, while whole ankyrin exhibits negative cooperativity (8) (Fig. 2). When the assay is reversed and the anion exchanger is the saturating ligand, interactions with both the 89-kDa domain and ankyrin are positively cooperative (Figs. 3 and 4). Positive cooperativity implies that the interaction of one ligand promotes the interaction of additional ligands. Thus, in membrane binding assays with the 89-kDa domain, the interaction of one 89-kDa domain promotes the interaction of a second 89-kDa domain to the same anion exchanger dimer. The two sites of interaction on the anion exchanger dimer are separate.
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Panel A contains a summary of the cooperative seen between ankyrin (protein 2.1) or the 89-kDa domain-binding domain of ankyrin and the anion exchanger dimer. Binding of the anion exchanger dimer to either ankyrin or the 89-kDa domain promotes the association of a second dimer with the corresponding complex. In the membrane assays, binding of an 89-kDa domain to an anion exchanger dimer promotes binding of a second 89-kDa domain, while binding of an ankyrin to a dimer inhibits the association of a second ankyrin to that dimer. Panel B depicts the likely consequences of combining these modes of cooperativity. The combination of reciprocal positive cooperativities involving the 89-kDa domain suggests that this domain can form a linear polymer with the anion exchanger dimer. In contrast, the positive cooperativity of the interaction of the dimer with ankyrin promotes the association of two dimers with ankyrin, but the negative cooperativity of the ankyrin interaction with the dimer inhibits the linkage needed to form a polymer.

and not within steric hindrance of each other. In the case of ankyrin, the interaction with erythrocyte membranes has negative cooperativity, indicating that interaction of one ankyrin inhibits the interaction of a second ankyrin to the same anion exchanger dimer. Ankyrin is a much larger molecule with a spectrin-binding domain and C-terminal region which may inhibit access to the second site on the anion exchanger dimer. When the 89-kDa domain or ankyrin is immobilized on latex beads this steric hindrance no longer applies; sites on the 89-kDa domain and ankyrin are being filled and steric hindrance between anion exchanger dimers will determine access to the sites (see Figs. 10 and 11).

The difference in salt sensitivities of ankyrin constructs D2-D3 and D3-D4 (Fig. 8) supports the presence of two separate ankyrin binding sites on anion exchanger dimers which differ in their dependence on electrostatic bonds. The interaction with the D3-D4 site is not salt sensitive, suggesting that this interaction is mediated by non-ionic contacts such as hydrogen bonding or hydrophobic surfaces. The salt insensitivity of the D3-D4 site is consistent with the stability of the ankyrin association with erythrocyte membranes at high concentrations of salt. In contrast, the subdomain two site was sensitive to high salt, suggesting that the interaction with the subdomain two site involved electrostatic contacts. Localization studies of the ankyrin site on the anion exchanger have identified two regions involved in the interaction (24, 25). One region was localized to the acidic N terminus; the other, to the region surrounding the flexible hinge. The highly charged nature of the N terminus suggests that this region may contain the site for subdomain two. If this is the case, then the D3-D4 site may involve the region surrounding the hinge (Fig. 10).

An alternative form of the anion exchanger missing the N-terminal 79 residues is expressed in kidney intercalated cells and has recently been shown to lack the ability to bind to construct D3-D4 (26). This result suggests that the D3-D4 site on the anion exchanger requires the first 79 amino acids. One possibility is that since both subdomains three and four are required for the D3-D4 interaction with the anion exchanger, one subdomain interacts with the first 79 amino acids and the other interacts with the hinge region. The location of the subdomain two site on the anion exchanger has not yet been evaluated. If this site also requires the N-terminal 79 amino acids, the truncated form of the anion exchanger may not interact with ankyrin. A lack of cytoskeletal attachment may allow for rapid turnover in the level of this truncated form of the anion exchanger in kidney membranes.

The interaction of ankyrin with the anion exchanger is regulated by alternative splicing and phosphorylation. An alternatively spliced form of erythrocyte ankyrin, which removes a portion of the C-terminal region, exhibits increased capacity and higher affinity for the anion exchanger in erythrocyte membranes (27-28). Both ankyrin and the cytoplasmic domain of the anion exchanger are substrates for protein kinase A and casein kinase. Phosphorylation by these kinases affect the capacity of the interaction of ankyrin with the cytoplasmic domain of the anion exchanger (29, 30). It will be of interest to define these regulatory effects in terms of the ankyrin-anion exchanger contacts resolved in this study.

Several predictions can be made from the finding of two distinct binding sites on both ankyrin and the anion exchanger. In erythrocyte membranes, the anion exchanger dimer is present at a 4-5-fold higher concentration than ankyrin. Deficiencies in the anion exchanger which reduce the ratio between the anion exchanger and ankyrin may allow two ankyrins to assemble on individual anion exchanger dimers. Since ankyrin is also bivalent, this interaction may lead to aggregation through the formation of \([\text{AE1}_{\text{K}}\text{-ANKL}]\) polymers. This process may occur naturally when hemichromes interact with the anion exchangers to form insoluble complexes (31), thereby reducing the level of free dimer in the membrane. Another route to this aggregation may be caused by denaturation or oxidation of...
the anion exchanger which may promote the formation of tetramers. Since ankyrin has two sites for the anion exchanger, such a situation could result in a [(AE1)_n-ANK]_m polymer. These polymerization events may be part of the senescence process in which the erythrocyte membrane becomes more rigid. Several clinical conditions have been described wherein alterations in anion exchanger result in abnormal membrane properties. These include Melasian ovocardiosis which is caused by a 9-amino acid deletion in the anion exchanger (32, 33) and a form of hereditary spherocytosis caused by reduced levels of the anion exchanger (34).

Interaction of the anion exchanger with ankyrin utilizes three of the four repeat subdomains, yet the anion exchanger is only one of seven known ankyrin-binding membrane proteins. How are sites for these seven targets accommodated on the repeat domains and how do they evolve? The simple hypothesis that the repeat domains are recognizing a common feature on the various targets is unlikely since the seven different families do not contain areas of homology in their cytoplasmic domains. While the possibility that repeat subdomains recognize a common tertiary structure cannot be discounted, a more likely hypothesis is that membrane proteins generated unique interactions with ankyrin through convergent evolution.

This evolved fit model (35) for the development of ankyrin interactions makes several predictions. First, if the membrane proteins evolved interactions with ankyrin, sites should not be restricted to individual repeat domains. This has been shown here with the site on D3-D4 for the anion exchanger. In addition, the Na+/K+-ATPase requires both 89-kDa domain and the spectrin-binding domain for its interaction with ankyrin (35). Second, because the anion exchanger utilizes three of the four repeat domains, the sites for other membrane proteins will likely overlap with the sites for the anion exchanger. This overlap may result in steric competition between different membrane proteins for sites on ankyrin. Currently known members of the ankyrin gene family, ankyrin_B, ankyrin_B, and ankyrin_C, contain conserved, but not identical membrane-binding domains (36-39). The conservation is strong enough that the three membrane-binding domains will likely interact with the complete set of ankyrin binding membrane proteins. However, the sequence differences may alter the relative affinities of these interactions. In the case of the anion exchanger, a membrane-binding domain construct of ankyrin_B is 8-fold weaker than the D3-D4 region of ankyrin_B in membrane binding assays (11). Differences in relative affinity may allow selective sorting of ankyrin isoforms with their appropriate membrane proteins.

Future work will hopefully elucidate the physiological role for multiple sites for the anion exchanger on ankyrin. An interesting question is whether the interaction of ankyrin with the anion exchanger precludes interactions with other ankyrin binding membrane proteins or whether ankyrin can assemble a complex of membrane proteins under specialized membrane domains.

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Addendum — While this work was in review, electron microscopy has observed ankyrin dependent aggregation of the anion exchanger into particles of a size consistent with a complex containing two anion exchanger dimers (40). This observation confirms our biochemical evidence which indicates that ankyrin can form a 4:1 complex with the anion exchanger through the use of two distinct sites in the membrane-binding domain.

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