Mapping of Ankyrin Binding Determinants on the Erythroid Anion Exchanger, AE1*

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The association of ankyrin with the AE1 anion exchanger contributes an essential function to the mechanical and viscoelastic properties of the erythrocyte and constitutes the best understood link between the plasma membrane and the underlying membrane skeleton. The AE1 binding domain of ankyrin consists of 24 tandem repeats of a 33-amino acid motif that is present on a wide variety of otherwise unrelated proteins. The experiments described in this paper are aimed at identifying the specific amino acid sequences in AE1 that comprise the ankyrin binding site. We have exploited a cell-free binding assay to quantify the binding affinity of anion exchangers and a recombinant fragment of ANK1, R13-H. Our previous study (Ding, Y., Casey, J. R. and Kopito, R. R. (1995) J. Biol. Chem. 269, 32201–32208) identified an essential role of the amino-terminal 79 AE1 residues in ankyrin binding. The present study extends these findings to show that these 79 amino acids, although necessary, are not sufficient for ankyrin binding. Using chimeras between AE1 and the closely related anion exchanger AE2, which does not bind ankyrin, we have defined a 40-residue region of AE1 between positions 155 and 195 that is also essential for ankyrin binding.

Ankyrins are heterobifunctional proteins that link the cytoplasmic domains of integral membrane proteins to the spectrin-based subcortical membrane skeleton (1). The ankyrin gene family includes ANK1 (“erythrocyte ankyrin” or ANK1), which is expressed in erythrocytes, and a small subset of CNS neurons (2, 3); ANK2 (“brain ankyrin” or ANKB) broadly expressed is expressed in erythrocytes, and a small subset of CNS neurons (2, 3); ANK3 (“general ankyrin” or ANK3) expresses in epithelia, mus-
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were also cloned into pRBG4. Δ156 was constructed from AE1 by deletion of codons 156–196 using megaprimer PCR (23, 24). The first round PCR used AE1 plasmid as template and a second primer flanking the 156–196 codon deletion. The resulting PCR product was used as a "megaprimer" in the second round PCR. The PCR fragment, carrying the 156–196 amino acid deletion, was digested with AgeI and SacI and then cloned into the corresponding site in AE1. All constructs were verified by sequence analysis. Chimera C8 was constructed by inserting PCR fragment of the codons 450–490 of AE2 into the AE1 site of Δ156. To make chimeras C1, an AE2 expression plasmid pBSL103 (21) was digested with XhoI and AccI to remove amino acids 1–290 and replaced by a PCR fragment containing the amino-terminal 79 amino acids of AE1. The remaining AE1/AE2 chimeras (C2–C6) were constructed using a PCR megaprimer mutagenesis strategy. Briefly, the first step was to generate an AE1 megaprimer by PCR using an AE1/AE2 chimeric primer, and in the second round PCR, pBSL103 was used as template and AE1 megaprimer was used as the forward primer. The PCR product, containing part of AE1 and the AE1/AE2 chimera junction region, was inserted into EcoRI and Stul sites of pSVK7 (pBSL103 containing a translationally silent deletion of the endogenous EcoRI site). Cardiac AE3 (cAE3) was generously provided by Dr. Gary Shull (University of Cincinnati). pYD9 (cardiac AE3 in pRBG4) was constructed by cutting out cardiac AE3 cDNA from pcDNA II with NotI and SpeI. The cardiac AE3 fragment was then filled with Klenow and cloned into the EcoRV site of pRBG4. All mutants were verified by sequence analysis and/or restriction analysis. Table I lists the details of the chimeras and constructs used in this study.

Purification and 125I Labeling of Recombinant Ankyrin Fragment R13-H—The expression construct for R13-H was kindly provided by Dr. Vann Bennett (Duke University). The purification and radioiodination of R13-H were as described previously (17).

Ankyrin Binding Assay—The ankyrin binding assays were performed as described previously (17). Briefly, transiently transfected HEK 293 cells were harvested and lysed in 0.1% Triton X-100 and 1% Nonidet P-40 in binding buffer without bovine serum albumin on ice for 15 min. The lysed cells were then centrifuged at 14,000 × g in an Eppendorf microfuge at 4 °C for 15 min. After removing 2 μl for protein assay, the supernatant was supplemented with bovine serum albumin to a final concentration of 1 mg/ml. The supernatant was recovered by immunoprecipitation with an antibody (5-288) raised to the amino terminus of AE1 (Fig. 2A) and AE3, which bound R13-H with high affinity (Kp = 59 nM), binding of R13-H to AE2 or AE3 was indistinguishable from the background value obtained for either vector-transfected extracts or for extracts from cells transfected with AE1m, an AE1 construct lacking the entire cytoplasmic domain (17). Likewise, no R13-H binding was observed with “cardiac” AE3, an AE3 variant expressed in heart and in retinal Müller cells that lacks the first seven exons from the amino-terminal cytoplasmic domain of AE3 (26, 28). These data suggest that the R13-H binding assay is appropriate to assess ankyrin-AE interaction and that AE3 and AE2 are poor ligands for ANK1.

Fig. 2A shows that AE1 and AE2 share a similar domain organization consisting of a highly conserved membrane domain (sequence identity, >80%) and a more diverged cytoplasmic domain. Consistent with the high degree of similarity, the membrane domains of AE1 and AE2 both catalyze the exchange of anions across the plasma membrane and are nearly indistinguishable in their transport properties (21). Despite the lower overall sequence identity between the cytoplasmic domains of AE1 and AE2, the sequences of these two proteins are clearly related and can be aligned with the aid of a computer algorithm (GAP) (29). Such alignment reveals the presence of eleven regions of similarity (with sequence identity, >50%) between AE1 and AE2 (Fig. 2A). The amino-terminal domain of AE2 is also ~220 residues longer than that of AE1. Using these homology domains as guides, we constructed a series of seven linear chimeras containing the carboxyl terminus of AE2 joined to the amino terminus of AE1 (Fig. 2B). The underlying assumption of these chimeras is that the homologous regions reflect regions of similar tertiary structure. Therefore, in each chimera the cDNAs were joined within such a region of high sequence identity (Fig. 2A). The amino-terminal domain of AE2 is also ~220 residues longer than that of AE1. Using these homology domains as guides, we constructed a series of seven linear chimeras containing the carboxyl terminus of AE2 joined to the amino terminus of AE1 (Fig. 2B). The underlying assumption of these chimeras is that the homologous regions reflect regions of similar tertiary structure. Therefore, in each chimera the cDNAs were joined within such a region of high sequence identity. Therefore, in each chimera the cDNAs were joined within such a region of high sequence identity.
homology, thereby minimizing the chances of gross structural distortion.

Expression of AE1/AE2 chimeras in HEK 293 cells was assessed by immunoblotting with an antibody that recognizes the carboxyl terminus of AE1 and AE2 (Fig. 3). All of the anion exchangers were expressed at similar levels, and all encoded functional anion plasma membrane exchangers. Binding of R13-H was determined for each of the chimeras (Fig. 4). The ankyrin fragment bound to chimeras C7, C6, and C5 with affinity indistinguishable from wild type AE1, suggesting that sequences in the carboxyl-terminal 174 amino acids of the cytoplasmic domain of AE2 do not account for the inability of this anion exchanger to bind ankyrin. By contrast, chimeras containing more than 278 amino acids from the cytoplasmic domain of AE2 (chimeras C1–C3) did not exhibit any significant ankyrin binding above background. Chimera C4 with 201 AE2 cytoplasmic residues exhibited weak ankyrin binding. Together, these data place a critical determinant for ankyrin binding between amino acids 176 and 304 of AE1.

The region between 176 and 304 of AE1 includes the proline-rich, putative hinge region at 175–190. Deletion of AE1 residues 156–195, which overlap this site, completely abolished detectable R13-H binding (Fig. 4), suggesting that this region either directly participates in ankyrin binding or that deletions in this region alter the protein’s conformation in such a way as to obscure a remote ankyrin binding determinant. As this region is rich in proline and glycine and is thought to form a

2 I. Sekler and R. Kopito, unpublished data.

![A](image1.png)  ![B](image2.png)

**Fig. 2.** Anion exchanger expression in transfected HEK cells. Immunoblot analysis of AE1, AE2, and AE1/AE2 chimeras (A) and AE3 (B). The mobilities of wild type anion exchangers are indicated.

![A](image3.png)

**Fig. 3.** Anion exchanger constructs used in this study. A, the graph in the upper panel indicates the average sequence homology between AE1 and AE2 for a sliding window of 10 residues. The relative positions of the regions exhibiting <50% identity is indicated graphically in the two lower panels. B, graphical representation of the anion exchanger mutants and chimeras used in this study.
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FIG. 4. Binding of mutant and chimeric anion exchanger to the ankyrin fragment, 125I-R13-H. The symbols are identified at the right. The data represent the mean ± S.E. of three independent determinations conducted in duplicate.

flexible hinge, we replaced the deleted residues with the corresponding sequence from AE2, which contains a similar number of helix-disrupting residues. That chimeric exchanger (C8) was also completely incapable of binding to R13-H. These data suggest that specific residues in the region 155–185 of AE1 directly participate in ankyrin binding.

DISCUSSION

The ankyrin repeat is a 33-amino acid motif present in a large and diverse number of otherwise unrelated proteins, where it is thought to participate in protein-protein interactions. The AE1 binding site of ANK1 is composed entirely of tandem ankyrin repeats arranged into four independently folded subdomains (27). The experiments described in this paper were aimed at dissecting the specific amino acid residues in AE1 that comprise the binding site for ankyrin. Our previous study identified a critical role of the amino-terminal 79 AE1 residues in ankyrin binding (17). The present study extends these findings to show that these 79 amino acids, although necessary, are not sufficient for ankyrin binding. Using chimeras between AE1 and the closely related anion exchanger AE2, which does not bind ankyrin, we have defined a 40-residue region of AE1 between positions 155 and 195 that is also essential for ankyrin binding.

In this study we have employed a quantitative cell-free binding assay to assess the interaction between the AE1–3 anion exchangers and the erythroid ankyrin isoform, product of the ANK1 gene. This assay confirms the conclusion from a previous study that suggested AE2 does not bind to ankyrin (30). However, the present data are inconsistent with the conclusions from that study in which an association between AE3 and ANK1 was reported. The present study differs from the previous in several important respects. In the previous study, ankyrin-AE interaction was assessed by the ability of the anion exchanger and an ankyrin fragment to form a stable complex at steady-state in cells cotransfected with both constructs (30). Therefore, those studies were limited by the inability to control either the concentrations of the two interacting species or the binding time. It is likely that those conditions could favor the formation of a complex between ankyrin and AE3 that reflects a binding affinity below the detection limit of the assay presented here. A second difference is that in the present study we have examined the interaction of anion exchangers with a fragment of ankyrin consisting of the carboxyl-terminal 12 ANK repeats (domains 3 and 4 (27)), whereas the previous study examined the interaction with an 89-kDa ankyrin fragment containing all 24 ANK repeats (domains 1–4). The binding of AE1 to ankyrin has recently been shown to involve two distinct, cooperative sites on ankyrin (31). One of these is apparently formed by determinants on domains 3 and 4, whereas the other requires determinants on domain 2 and 3 (31, 32). The present data, therefore, could reflect the possibility that high affinity AE3 binding to ankyrin may require additional determinants present within the first 12 repeat units.

In the present study, we have investigated the interaction of ANK repeat domains 3 and 4 and chimeras between AE1 and AE2, which does not interact with ANK1 ankyrin in any assay. The rationale for the construction of AE1-AE2 chimeras is premised on the assumption that despite the relatively low overall sequence identity between the cytoplasmic domains of the two anion exchangers, the presence of interspersed regions of high sequence homology reflects an overall similarity in the secondary and tertiary structures of these two proteins. This assumption is supported by computer-assisted secondary structure analysis (29). The junctions between AE1 and AE2 in the chimeras were placed within regions of high homology, where the alignment between the two proteins was unambiguous.

Thus, chimera C1 was constructed with the amino-terminal 291 residues of AE2 replaced with the 79 residues from the amino terminus of AE1 in order to preserve the relative position of those residues. The absence of high affinity binding of R13-H to chimera C1 strongly suggests that the 79 residues, although essential, are not sufficient for ankyrin binding.

Our data indicate a sharp drop in ankyrin binding affinity as the chimeras become more AE2-like, with the “threshold” between AE1 residues 176 and 304. This finding is consistent with the existence of a critical determinant for ankyrin binding lying within this region that has been proposed to include a putative flexible hinge (16, 18). Previous studies have implicated this segment of AE1 in ankyrin binding (19, 20). Therefore, this domain could be important for ankyrin binding either through direct interaction with ankyrin or by providing conformational flexibility that permits the correct alignment of AE1 relative to ankyrin. The lack of detectable ankyrin binding to chimera C8, in which residues 156–190 were substituted with similarly flexible proline-rich segment of AE2, suggests that ankyrin may interacts directly with this region. Additional chimeras and substitutions in this region will be helpful in further defining the nature of this site.

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