The Membrane-binding Domain of Ankyrin Contains Four Independently Folded Subdomains, Each Comprised of Six Ankyrin Repeats*

(Received for publication, May 3, 1993, and in revised form, July 7, 1993)

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Ankyrin repeats are a 33-amino acid motif present in a number of proteins of diverse functions including transcription factors, cell differentiation molecules, and structural proteins. This motif has been shown to mediate protein interactions in the case of ankyrin as well as several other repeat-bearing proteins. In ankyrin, 24 tandemly arrayed repeats are arranged to form a globular, membrane-binding domain. This report provides evidence that the repeats in this domain fold into four independently folded subdomains of six repeats each. Limited proteolytic digestions of defined regions of the membrane-binding domain identified protease-sensitive sites, which divided this domain into subdomains of approximately six repeats each. Hydrodynamic measurements and circular dichroism spectroscopy of expressed subdomains confirmed that these six-repeat regions exist as folded, globular structures. The requirement of a complete set of six repeats for proper folding was determined using a series of protein constructs, which sequentially deleted repeats from the last subdomain. Deletion of even one repeat resulted in a 40% loss of α-helicity. Deletions removing three or more repeats abolished the helical signal completely. The spherical shapes of the intact domain and of the subdomains (inferred from hydrodynamic values) suggest that the four subdomains are organized in either a tetrahedral or square planar configuration. Two six-repeat subdomains were found to be required for high affinity association with the anion exchanger, suggesting that at least some of the protein interactions mediated by ankyrin repeats involve multiple subdomains.

Ankyrin repeats are a 33-amino acid motif typically present in tandem arrays of four to seven copies and are contained in a collection of proteins of diverse localization and function (reviewed in Michaely and Bennett (1992)). Repeat-bearing proteins occur in species ranging from Eubacteria to man and in cellular localizations ranging from secreted proteins to structural proteins. Functions of these proteins vary widely and include α-latrotoxin of black widow spider venom, proteins that control cell differentiation, transcription factors, and the cytoskeletal protein, ankyrin.

Ankyrin repeats of several repeat-bearing proteins have been implicated in interactions with apparently unrelated target proteins and, thus, the ankyrin repeats have been proposed to function as a generalized protein binding motif (Michaely and Bennett, 1992). The NF-κB transcription factor system includes several proteins containing ankyrin repeats such as the p105 precursor of the p50 subunit of NF-κB (Lux et al., 1990; Kieran et al., 1990), IκBα (MAD3) (Haskill et al., 1991), IκBβ (rel-associated pp40) (Davis et al., 1991b), IκBγ (Inoue et al., 1992a), bcl3 (Ohno et al., 1990), and cactus (Geisler et al., 1992). The repeats in p105 (Henkel et al., 1992), IκBβ (Inoue et al., 1992b), and bcl3 (Wulczyn et al., 1992; Bours et al., 1993) have been shown to interact directly with p50 subunits. A binding function has also been identified for the four repeats of the β subunit of the interferon-induced transcription factor, GABP. These ankyrin repeats are required for association with the α subunit of GABP and enhance the specificity of the DNA binding activity of the α subunit (Thompson et al., 1991; LaMarco et al., 1991).

Ankyrin repeats are involved in many of the protein interactions of ankyrins. Ankyrins are a multifaceted family of proteins proposed to function as adaptors between the spectrin-based, membrane skeleton and membrane proteins, which include both ion channels and cell adhesion molecules (reviewed in Bennett and Gilligan (1993)). Ankyrins have two binding domains as follows: a 62-kDa domain containing the spectrin binding site and a domain of 89-95 kDa, which is involved in membrane interactions. The membrane-binding domains are comprised of 24 tandemly arrayed ankyrin repeats and are folded into a nearly spherical structure (Davis and Bennett, 1990a). The number of repeats in ankyrin was initially reported to be 22 (Lux et al., 1990); however, using less stringent homology criteria and the exon-intron map of erythrocyte ankyrin (Tse, 1990), 24 repeats can be resolved (Michaely and Bennett, 1992). The repeats are necessary and sufficient for association of ankyrin with the anion exchanger (Davis et al., 1991b; Davis and Bennett, 1990a), the voltage-dependent sodium channel (Srinivasan et al., 1992), and the nervous system cell adhesion molecule, ABGP186, which is related to L1 and neurofascin (Davis et al., 1993). The membrane-binding domain is also required for association with the Na⁺/K⁺ ATPase, although this domain alone is not sufficient for high affinity binding (Davis and Bennett, 1990b).

Unresolved issues include identifying how many ankyrin repeats are required to form a stable structure and determining how the arrangement of repeats relates to their observed binding activities. This study provides evidence that individual ankyrin repeats are not capable of folding independently and that the 24 repeats of erythrocyte ankyrin fold cooperatively into four subdomains of six repeats each. Furthermore, two six-repeat subdomains were found to be required for high

* This work was supported in part by National Institutes of Health Grant 537DK29808. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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affinity association with the anion exchanger, suggesting that at least some protein interactions are mediated by multiple subdomains. Finally, using the hydrodynamic properties of the subdomains, we propose a model for the compact arrangement of the subdomains in the membrane-binding domain.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free Na\(^{22}\)I was obtained from Amersham Corp. and I\(^{125}\)-labeled Bolton-Hunter reagent, from ICN Radiochemicals. Disopropyl fluorophosphate, leupeptin, pepstatin A, dithiorthio, phenylmethylsulfonyl fluoride, benzamidine, isoandrosterone, NaF, thiglycolic acid, n-octylglucoside, Tween 20, o-chymotrypsin, bovine serum albumin, ovalbumin, and cytochrome c were from Sigma. Blue dextran and all chromatography matrices were obtained from Pharmacia LKB Biotechnology Inc. Staph V8 protease was purchased from Pierce Chemical Co., DNase I from U.S. Biochemical Corp., and Taq polymerase from Perkin-Elmer Cetus Instruments. Isopropylthiogalactopyranoside was purchased from ICN Biochemicals. The pGEMEX expression vector was from Promega. Triton X-100 was from Boehringer Mannheim. Nitrocellulose paper was from Bio-Rad and Immobilon paper from Millipore.

Methods

Bacterial Expression of Protein Constructs Containing Defined Regions of the Ankyrin Sequence—Protein expression was performed using a T7 expression system (Studier and Moffatt, 1986; Davis et al., 1991a). Briefly, polymerase chain reactions were used to amplify specific regions of the ankyrin DNA sequence. These polymerase chain reaction products were then cloned into the pGEMEX vector (Promega) such that the ankyrin sequences were in-frame with the start codon of the gene 10 promoter. These constructs were designed to code for only an added methionine, alanine, and serine at the N terminus of the ankyrin sequences. Recombinant plasmids were then transfected into an E. coli transformant to express the protein. Cell lysates were subjected to SDS-PAGE and Western blots probed with polyclonal antibody raised against erythrocyte ankyrin. In addition, constructs R20-H through R24-H were used as standards. Peak fractions of the gel filtration runs used to determine the Stokes radii were dialyzed into the above buffer and used as the protein samples for the sedimentation runs. Fractional coefficients were calculated using the following equations.

\[
M_i = \frac{5\pi N_i R_i s_{20,w}}{1 - v_{p,M}} \\
I/f_i = R_i \frac{4\pi N_i}{3 M_i (1 + k/\rho)}
\]

(Eq. 1)

(Eq. 2)

Partial specific volumes for this calculation were estimated from the amino acid sequence of the various protein constructs (Cohn and Edsall, 1945).

Procedures

SDS-PAGE\(^1\) was performed on exponential (3.5-17%) polyacrylamide gels (Davis and Bennett, 1984). Blot binding of I\(^{125}\)-labeled cytoplasmic domain of the red cell anion exchanger to immobilized ankyrin constructs (Davis and Bennett, 1990a) and the solution binding of I\(^{125}\)-labeled R13-H construct to ankyrin-depleted, inside-out erythrocyte vehicles (Bennett and Stenbuck, 1980) were performed essentially as previously described. The native 88-kDa membrane-binding domain was isolated from chymotryptic digests of erythrocyte ankyrin as previously described (Davis and Bennett, 1990a).

RESULTS

Identification of Subdomain Structure in the Membrane-Binding Domain of Ankyrin by Limited Proteolysis—The presence of subdomains within the membrane-binding domain of erythrocyte ankyrin was identified by protease digestion. Staph V8 protease cleaves the isolated domain at a cleavage site at glutamate 402 (Davis and Bennett, 1990a). This site is near the border between the 12th and 13th repeats and divides the 24 repeats into two subdomains of 12 repeats each. These N-terminal and C-terminal 12-repeat subdomains were expressed in bacteria and subsequently purified to homogeneity using gel filtration and ion exchange chromatography (Fig. 1). The 12-repeat domains are likely to be in native configurations since they encompass proteolytically defined regions and are compactly folded based upon hydrodynamic measurements (see below and Table I). The C-terminal 12-repeat construct was further characterized to be in a native state both structurally based upon circular dichroism data (see below and Fig. 3) and functionally based upon its affinity for membrane binding sites (see below and Fig. 8).

The 12-repeat subdomains were subjected to a further round of proteolysis (Fig. 1). Staph V8 digestion of construct N-R12 (containing the N-terminal 10 residues and the first 12 repeats) produced a 24-kDa polypeptide with the same N-terminal sequence as the N-R12 construct. The apparent molecular mass of the product indicated that it contained approximately six to seven repeats. Chymotryptic digestion of the R13-H construct (containing repeats 13-24 and a 32-residue “hinge” region after the last repeat) produced three proteolytic products. The highest molecular mass product is likely to be the result of cleavage at a previously identified site at phenylalanine 795 (Davis and Bennett, 1990), which removes the hinge region. The lower two products have identical N termini and result from hydrolysis after leucine 587, near the border between the 18th and 19th repeats. The smaller of these two lower bands is likely derived from cuts at both leucine 587 and phenylalanine 795. As illustrated in Fig. 1D, the positions of these proteolytic sites divide the membrane-binding domain into four subdomains of approximately six repeats each.

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; R\(_i\), Stokes radius.

Definition of the Minimum Folding Unit of Ankyrin Repeats—Circular dichroism (CD) spectra of bacterially expressed constructs were used to determine how many repeats...
are required to form a folded domain. CD spectroscopy was used to monitor folding, because similar amino acid sequences typically produce similar secondary and tertiary structures, and CD spectroscopy can directly probe the degree of secondary structure. Thus, if these repeat constructs are correctly folded, they should produce similar CD spectra. This prediction proved correct for protein constructs containing 6, 12, and 24 repeats (see Figs. 2 and 3). These constructs included N-R6 (containing the first 10 amino acids and the first six repeats), CR19-H (the 13 amino acids from the chymotryptic cleavage site to the start of the 19th repeat), repeats 19–24, and the 32-amino acid hinge region at the end of repeat 24), R19-H (the last six repeats and the hinge region), and the 12-repeat subdomain, R13-H (the last 12 repeats and the hinge region). Unfortunately, expressed subdomains, which encompassed the N-terminal 12 repeats (N-R12), repeats 7–12 (R7-R12), and repeats 13–18 (R13-R18) were not soluble in the low salt conditions required for CD measurements and could not be examined. However, the constructs that were soluble exhibited remarkably similar spectra with calculated helicities clustered between 16 and 20% (Fig. 3).

A nested series of protein constructs was prepared with sequential deletions of repeats in order to determine how many repeats are required to maintain native folding (Fig. 4). This series was derived from the six-repeat construct, CR19-H, which encompassed the region corresponding to the major chymotryptic fragment of the R13-H construct (Fig. 1). The first deletion construct, R19-H, removed the 13-amino acid region from the chymotrypsin site to the start of the 19th repeat. The remaining constructs were prepared with N-terminal deletions of one repeat (R20-H), two repeats (R21-H), three repeats (R22-H), four repeats (R23-H), and five repeats (R24-H) (see Fig. 4 for diagrams illustrating the regions encompassed by each construct).

Constructs CR19-H and R19-H, which retain a complete set of six repeats, exhibited native circular dichroism spectra (Fig. 5). Constructs R20-H and R21-H, which are missing one and two repeats, respectively, suffered a loss of signal corresponding to a greater than 40% loss of α-helical content. When three repeats were removed (construct R22-H), the helical signal was lost completely; the spectra converted to that of a random coil (Fig. 5).

Constructs R20-H and R21-H, which contain five and four repeats, respectively, retain some helical signal. However, these polypeptides are less compactly folded than the six-repeat constructs based on their behavior on gel filtration. Constructs R20-H and R21-H have Stokes radii (2.4 and 2.6 nm, respectively) greater than that of the CR19-H and R19-H constructs (2.4 and 2.3 nm, respectively) despite having smaller molecular weights (data not shown). The loss of helical content and increased Stokes radii of constructs R20-H and R21-H could be caused either by a dynamic equilibrium between a folded and unfolded state or by an unfolding of a helical portion of the repeat sequence normally stabilized by the 19th repeat. While it is possible that deletions of individual repeats within a six-repeat domain could be tolerated, these results strongly suggest that the domain structure of the repeats in ankyrin utilizes folding units of six ankyrin repeats.

Use of Hydrodynamic Properties to Estimate Molecular Shape and Oligomerization State of the Subdomains in Solution—The native membrane-binding domain has been previously shown to have a frictional coefficient of 1.2, indicating a nearly spherical shape (Davis and Bennett, 1990a). Sedimentation coefficients were estimated by rate zonal sedimentation on sucrose gradients. Stokes radii were determined by gel filtration using Superose 12. These values were then used to calculate frictional coefficients for the individual six-repeat subdomains (Table I). Calculated frictional coefficients clustered between 1.2 and 1.3, indicating that these subdomains are also nearly spherical.

Physical measurements of constructs R7-R12 and N-R12 suggest that subdomains can self-associate, perhaps as the result of similar contacts utilized in assembly of the normal organization of four subdomains. The 12-repeat construct, N-R12, formed a dimer in solution with hydrodynamic properties nearly identical to the native 24-repeat domain. The sedimentation coefficient of the R7-R12 construct was not determined due to low solubility in 1 M NaCl. However, gel filtration runs
Multirepeat Folding Units of Ankyrin

### TABLE I

| Construct | Sedimentation | Stokes radius | Calculated M,\(^\text{c}\) | Actual M,\(^\text{c}\) | Solution state | f/f₀ |
|-----------|---------------|---------------|-----------------|-----------------|----------------|------|
| 89 kDa    | 5             | 39            | 82              | 89              | Monomer        | 1.2  |
| N-R12     | 5.4           | 38.5          | 91.6            | 43.7            | Dimer          | 1.2  |
| R13-H     | 3             | 33            | 43              | 45.6            | Monomer        | 1.3  |
| N-R6      | 1.5           | 25            | 18.9            | 22.2            | Monomer        | 1.3  |
| R7-R12    | ND            | 39.5          | ND              | 21.9            |                |      |
| R13-R18   | 2             | 24.5          | 21.3            | 21.6            | Monomer        | 1.3  |
| R19-H     | 2             | 23.4          | 23.6            | 24.7            | Monomer        | 1.2  |
| CR19-H    | 2.2           | 24.2          | 26.4            | 25.9            | Monomer        | 1.3  |

* Sedimentation values are expressed in Svedberg units.
* Stokes radii are shown in Angstrom units.
* Molecular masses are shown in kilodaltons.
* ND, experiments not done.

#### FIG. 2

Coomassie Blue-stained SDS gels of purified protein constructs containing sets of six and 12 repeats. Purified protein constructs containing six (N-R6, R7-R12, R13-R18, and R19-H) and 12 (N-R12 and R13-H) repeats were electrophoresed on SDS-polyacrylamide gel and stained with Coomassie Blue. The protein constructs were uniformly loaded on the gel with 1.5 μg of protein in each lane. The name of the construct loaded is used as the designation for each lane with the regions encompassed by each construct illustrated in panel B. The region marked \( \text{N} \) refers to the first 10 amino acids from the methionine at the N terminus to the start of the repeat region. The regions with \( \text{numbers} \) refer to the individual ankyrin repeats. The region marked \( \text{H} \) indicates the hinge region, which contains 32 amino acids from the end of the repeat region to the chymotrypsin site at residue 827. The exact residues encompassed by each construct are as follows: construct N-R12 contains residues 1-402; construct R13-H, residues 403-827; construct N-R6, residues 1-204; construct R7-R12, residues 205-402; construct R7-R12, residues 403-600; and construct R19-H, residues 601-827.

In 1 M NaBr yielded a Stokes radius corresponding to the size of the entire membrane-binding domain, suggesting that this construct may be a tetramer in solution.

The spherical shapes of the intact domain and the subdomains suggest that the four subdomains are organized in either a tetrahedral or a square planar configuration. Linear arrangements of subdomains are not likely, since the resulting extended structure would be expected to have a higher frictional ratio (Fig. 6).

### FIG. 3

Circular dichroism spectra of polypeptides containing sets of six, 12, and 24 repeats. Upper panel, circular dichroism spectra were measured as described under “Methods” for constructs N-R6, CR19-H, R19-H, R13-H, and the native membrane-binding domain. As discussed in the text and in the legend to Fig. 2, constructs N-R6, CR19-H, and R19-H encompass individual six-repeat subdomains, while construct R13-H contains both of the last two subdomains (repeats 13–24). The native membrane-binding domain contains all 24 repeats and is designated by 89kD in reference to its 89-kDa molecular mass. Lower panel, the percent \( \alpha \)-helices corresponding to each construct were estimated from the measured ellipticities at 220 nm using the algorithm of Chen and Yang (1971).
Multirepeat Folding Units of Ankyrin

Fig. 4. Coomassie Blue-stained SDS gel of a nested series of recombinant polypeptides with N-terminal deletions of repeats within the region corresponding to the chymotryptic fragment of the R13-H construct. A nested deletion series of protein constructs were designed through the proteolytically defined region of the R13-H construct. Following purification of these constructs, 3 μg of each protein was electrophoresed on an SDS-polyacrylamide gel and stained with Coomassie Blue (panel A). The name of the construct loaded in each lane is used as the lane designation with the regions encompassed by each construct illustrated in panel B. N refers to the first 10 amino acids, the regions with numbers refer to the repeat number, and H refers to the 32-amino acid hinge region. The native membrane-binding domain and the R13-H domain are shown for reference. The exact residues encompassed by each construct are as follows: construct CR19-H contains residues 588-827; construct R19-H, residues 601-827; construct R20-H, residues 634-827; construct R21-H, residues 667-827; construct R22-H, residues 700-827; construct R23-H, residues 733-827; and construct R24-H, residues 766-827.

(AE1) Requires Two Six-repeat Subdomains—Previous work has shown that while repeats 22 and 23 are required for the interaction of ankyrin with the anion exchanger, the activity of ankyrin repeat constructs decreased as the number of repeats was reduced below 11 (Davis et al., 1991b). Since loss of activity in binding was correlated with the simultaneous loss of helical content as measured by CD spectroscopy, loss of secondary structure was interpreted to be the cause of the loss of binding activity (Davis et al., 1991b). The six-repeat constructs R19-H and CR19-H contain repeats 22 and 23, displayed native circular dichroism spectra, and formed compact, globular structures. Nevertheless, these polypeptides did not exhibit high affinity binding to the anion exchanger. These constructs were at least 5-fold less active than the 12-repeat construct, R13-H, in the ability to compete with native ankyrin for anion exchanger sites on ankyrin-depleted inside-

Fig. 5. Circular dichroism spectra of the deletion series shows that native-like folding requires a complete set of six repeats. CD spectra for the deletion series (CR19-H, R19-H, R20-H, R21-H, R22-H, R23-H, and R24-H) were measured according to the protocol described under “Methods” and are displayed in the upper panel. The lower panel contains the calculated percent α-helix (Chen and Yang, 1971). Constructs CR19-H and R19-H, which differ only by a 13-amino acid extension at the N terminus of CR19-H, have virtually overlapping spectra and a correspondingly equivalent percent α-helicity. Constructs R19-H and R20-H show a 41 and 48% loss of α-helical content, respectively. Deletion constructs R22-H, R23-H, and R24-H, which contain three or fewer repeats, display a nonhelical spectra with undetectable levels of α-helix.

Fig. 6. A model for the arrangement of subdomains in the membrane-binding domain. Potential models for the arrangement of the four six-repeatsubdomains in the membrane-binding domain must account for both the approximately spherical shapes of the individual subdomains as well as the near spherical shape of the whole domain. Models that can accommodate spherical objects into compact units include square planar and tetrahedral configurations. Linear arrangements are unlikely, since such an extended arrangement would not produce small frictional coefficients.
out, erythrocyte membrane vesicles (data not shown). Also, in direct binding assays, association of $^{125}$I-labeled R19-H or CR19-H protein constructs with membrane vesicles could not be detected (data not shown).

A qualitative blot binding assay illustrated the relative ability of the cytoplasmic domain of the anion exchanger to interact with the 6-, 12-, and 24-repeat constructs. (Fig. 7). Individual six-repeat constructs failed to interact with $^{125}$I-labeled cytoplasmic domain in contrast to the 12-repeat R13-H construct, intact membrane-binding domain, and native erythrocyte ankyrin, all of which interacted strongly. This result suggests that the interaction pocket for the cytoplasmic domain of the anion exchanger requires sites on both the third and fourth subdomains of the membrane-binding domain.

A role for all four subdomains in association with the anion exchanger is suggested by comparison of the 12-repeat R13-H construct and the native 24-repeat domain in membrane binding assays (Fig. 8). Values for half-maximal binding of $^{125}$I-labeled R13-H construct and intact membrane-binding domain were nearly identical in measurements of the association of these proteins with inside-out erythrocyte membranes. However, Hill plots revealed a subtle difference in behavior of the 12- and 24-repeat polypeptides. The Hill plot for the 12-repeat subdomain exhibited a single line with a slope of 1.3, while plots for the 24-repeat domain were biphasic with a slope of 1.2 at low concentrations and a slope of 2.1 at higher concentrations. These results suggest that the N-terminal half of the membrane-binding domain has some role in mediating cooperativity in binding to membrane sites.

**DISCUSSION**

This report provides the first evidence that ankyrin repeats do not fold independently but rather require multiple repeats to form folded domains. In the case of ankyrin, the 24 repeats of the membrane-binding domain are organized into four folded subdomains of six repeats each. The existence of six-repeat subdomains was first suggested by proteolytic diges-

tions of various repeat regions of the membrane-binding do-

main (Fig. 1). Deletions that reduced the number of repeats in the last subdomain disrupted the folding of this domain, suggesting a requirement of six repeats for the folding of the other subdomains as well. Hydrodynamic properties and circular dichroism spectra of six-repeat constructs determined that these regions have compact, globular structures with levels of $\alpha$-helix comparable with that of the native 24-repeat domain. We present a model for the compact arrangement of these six-repeat subdomains in the membrane-binding domain as either a tetrahedral or square planar array (Fig. 6).

Not just any set of six ankyrin repeats appear to be capable of folding, since previous work has shown that a six-repeat construct offset by one repeat from the last six-repeat domain has sharply reduced $\alpha$-helical signal in solution (Davis et al., 1991a). The requirement of ordered arrays of repeats for the folding of repeat domains suggests that individual repeats have unique features whose specific interactions lead to the formation of folded domains.

Participation of multiple repeats in the formation of properly folded domains is not unique to ankyrin repeats. Several repeated amino acid motifs have been identified, which appear to utilize multiple copies to form properly folded structures. These motifs are normally present in tandem arrays and are typically shorter than individually folded motifs such as the immunoglobulin and fibronectin domains. Examples include the 24-amino acid repeat of the $\alpha_2$-glycoprotein (Takahashi et al., 1985), the 18-amino acid repeat of the MAP2 (Lewis et al., 1988) and tau proteins (Lee et al., 1988), and the 11 and 22 amino acid repeats of the apolipoproteins (Boguski et al., 1986). The crystal structure of apolipoprotein E (Wilson et al., 1991) has been solved and has clearly shown that the 11- and 22-amino acid repeats fold cooperatively to form a single helical bundle.

The majority of ankyrin repeat-bearing proteins contain tandem arrays of four to seven repeats (Michael and Bennett, 1992), which suggests that, like the repeats in ankyrin, the repeat arrays in these proteins fold cooperatively to form

**FIG. 7. Binding of the cytoplasmic domain of the anion exchanger requires multiple subdomains.** The 82-kDa protein construct of brain ankyrin, native erythrocyte ankyrin, and domains of erythrocyte ankyrin were electrophoresed on SDS-polyacrylamide gels and either stained with Coomassie Blue (panel A) or electrophoretically transferred to nitrocellulose paper. After blocking the nitrocellulose paper with 40 mg/ml bovine serum albumin solution in a buffer containing 10 mM sodium phosphate, 100 mM NaCl, 0.2% Triton X-100, 0.5% Tween 20, and 1 mM of each of NaEDTA, NaN3, and dithiothreitol (pH 7.4), $^{125}$I-labeled anion exchanger cytoplasmic domain (47,000 cpm/pmol) was added to 20 nM and incubated with moderate shaking overnight at 4 °C. Panel B shows an autoradiogram of this blot. For the autoradiogram in panel C, 1 µM cold anion exchanger cytoplasmic domain was added in conjunction with the $^{125}$I-labeled cytoplasmic domain to assess nonspecific binding. 82kD is protein construct derived from the brain ankyrin gene product and contains the region corresponding to the last 19 repeats and a small portion of the spectrin-binding domain (Davis et al., 1993). Ankl is native erythrocyte ankyrin. 82kD is the native membrane-binding domain of erythrocyte ankyrin. 62kD is the native spectrin-binding domain of erythrocyte ankyrin. The remaining lane designations are the names of the individual repeat constructs previously described in the legend to Fig. 2.
two duplication events beginning with a gene encoding a possibility, the 24-repeat domain may have been formed by contact sites formed between individual subdomains could action sites on the membrane-binding domain. Moreover, affinity interaction with the anion exchanger (Fig. that at least some of the proteins that associate with ankyrin binding sites can be formed by various combinations of subdomains, additional membrane protein binding sites presumably resulted from interactions that involved contacts with the surface of the tetrameric domain and possibly other portions of the ankyrin molecule. Of future interest will be the identification of any membrane proteins that are capable of interacting with individual six-repeat domains, since these sites may represent the most ancient binding sites and may provide insight into the original interactions of ankyrin.

Acknowledgments—We gratefully acknowledge Lydia Davis for providing KI stripped inside-out erythrocyte membranes and purified cytoplasmic domain of erythrocyte anion exchanger. We thank Steve Lambert for many stimulating discussions and for providing portions of the sequence of erythrocyte ankyrin for the construction of the various protein constructs. We are indebted to Judy Phelps for performing microsequencing of the myriad protein constructs used in this work.

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