Isoform Specificity of Ankyrin-B

A SITE IN THE DIVERGENT C-TERMINAL DOMAIN IS REQUIRED FOR INTRAMOLECULAR ASSOCIATION*

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Khadar M. Abdi, Peter J. Mohler, Jonathan Q. Davis, and Vann Bennett

From the †Howard Hughes Medical Institute, and Departments of Cell Biology, Biochemistry, and Neurosciences, Medical Center, Duke University, Durham, North Carolina 27710 and the ‡Department of Pathology, Medical Center, Vanderbilt University, Nashville, Tennessee 37232

Ankyrins contain significant amino acid identity and are co-expressed in many cell types yet maintain unique functions in vivo. Recent studies have identified the highly divergent C-terminal domain in ankyrin-B as the key domain for driving ankyrin-B-specific functions in cardiomyocytes. Here we identify an intramolecular interaction between the C-terminal domain and the membrane-binding domain of ankyrin-B using pure proteins in solution and the yeast two-hybrid assay. Through extensive deletion and alanine-scanning mutagenesis we have mapped key residues for interaction in both domains. Amino acids 1597EEE1599 located in the ankyrin-B C-terminal domain and amino acids Arg79/Arg40 located in ANK repeat 1 are necessary for inter-domain interactions in yeast two-hybrid assays. Furthermore, conversion of amino acids EED1597 to AAA1599 leads to a loss of function in the localization of inositol 1,4,5-trisphosphate receptors in ankyrin-B mutant cardiomyocytes. Physical properties of the ankyrin-B C-terminal domain determined by circular dichroism spectroscopy and hydrodynamic parameters reveal it is unstructured and highly extended in solution. Similar structural studies performed on full-length 220-kDa ankyrin-B harboring alanine substitutions, EED1597AAA1599, reveal a more extended conformation compared with wild-type ankyrin-B. Taken together these results suggest a model of an extended and unstructured C-terminal domain folding back to bind and potentially regulate the membrane-binding domain of ankyrin-B.

Ankyrins are a family of membrane adaptor proteins required for the localization of diverse ion channels, transporters, calcium-release channels, and cell adhesion molecules to specialized membrane domains (1). The ankyrin family is comprised of three genes, ANK1, ANK2, and ANK3, that encode ankyrin-R, ankyrin-B, and ankyrin-G polypeptides, respectively. Human ankyrin-R mutations are a major cause of hereditary spherocytosis, a defect in proteins that connect the membrane skeleton to the lipid bilayer (2). Ankyrin-B polypeptides are required for the localization of InsP3R2 receptor, Na/Ca exchanger, and Na/K-ATPase to transverse-tubule/sarcoplasmic reticulum membranes in heart (3). In addition, loss-of-function mutations in ankyrin-B cause a stress-induced cardiac arrhythmia syndrome in humans (3, 4). Finally, ankyrin-G is required for the assembly of βIV-spectrin, neurofascin, and voltage-gated sodium channels at excitable membranes in the nervous system (5) and for targeting voltage-gated sodium channels in heart (6).

An important unresolved question in understanding ankyrin function is the molecular basis for ankyrin specificity in vivo. Ankyrin-B and ankyrin-G are closely related in amino acid sequence (67% amino acid identity between their membrane-binding, spectrin-binding, and death domains) and are commonly co-expressed in many cell types. Nevertheless, ankyrin-B and ankyrin-G interact with different membrane proteins and perform distinct functions in vivo. For example, ankyrins-B and -G are both expressed in cardiomyocytes. However, ankyrin-B is required for the localization of the InsP3R receptor, Na/K-ATPase, and Na/Ca exchanger to specialized sites on T-tubules, whereas ankyrin-G activity is required for targeting of the cardiac voltage-gated sodium channel (Nav1.5) (6). Furthermore, only transfection of ankyrin-B into ankyrin-B null neonatal cardiomyocytes can rescue the localization of the InsP3R receptor, whereas ankyrin-G is inactive (7).

Studies with ankyrin-B/G chimeras support a key role for the highly divergent ankyrin-C-terminal regulatory domain in defining specific functions of ankyrin-B and ankyrin-G in cardiomyocytes (7). The C-terminal domain could regulate ankyrin specificity via interactions with other proteins, or through inter-domain interactions. To date Hsp40 and obscurin have been shown to bind the ankyrin-B C-terminal domain (8, 9). Evidence for inter-domain interactions of the regulatory domain has come from studies of ankyrin-R. Ankyrin-R 2.2, an alternatively spliced variant missing 162 residues in the regulatory domain, binds to erythrocyte-spectrin and the anion exchanger with higher affinity than the original ankyrin-R 2.1 (10). The peptide corresponding to the missing residues bound directly to ankyrin-R 2.2 and inhibited binding of ankyrin-R 2.2 to spectrin and the anion exchanger (11).

Here we provide evidence for a functionally important interaction between the C-terminal and membrane-binding domains of ankyrin-B. We identify sites of interaction between these domains and have generated mutations in 220-kDa ankyrin-B leading to loss of this inter-domain interaction as well as activity in cardiomyocytes. Structural analysis of the C-terminal domain reveals an extended conformation capable of contacting the membrane-binding region of ankyrin-B. Together these results provide evidence for a role of intramolecular interactions in driving ankyrin-B specific function, and offer a potential mechanism for regulation of ankyrin-B activity.

MATERIALS AND METHODS

Hydrodynamic Measurements—The Stokes radii and sedimentation coefficients of full-length ankyrins and the C-terminal domain were determined as described previously (12).
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Immunofluorescence—Neonatal cardiomyocytes were prepared, transfected, and analyzed as described previously (7, 9). Images were collected using a Zeiss 510 Meta confocal microscope (40 power, oil objective, 1.4-numerical aperture (Zeiss)) using imaging software from Zeiss.

Yeast Two-hybrid Assay—The Gal4 yeast two-hybrid system was used to test direct binding between domains of 220-kDa ankyrin-B. Fragments were PCR-amplified and inserted either in the pACT2 (GAL-4 activation domain vector) or pAS2-1 (GAL-4 DNA-binding domain vector). All constructs were completely sequenced and were free of mutations. Fusion protein expression in yeast was confirmed by immunoblot analysis using either commercially available antibodies to GAL-4 fusions (Clontech) or affinity-purified antibodies to ankyrin-B. Plasmids were co-transformed into AH109 yeast (Clontech) and exhibited growth on -Ade/His/Leu/Trp media were considered positive for interaction.

Ankyrin-B Constructs and Mutagenesis—cDNAs for the membrane-binding domain (aa 1–959), spectrin-binding domain (aa 862–1444), death domain (aa 1445–1555), and C-terminal domain (aa 1556–1840) were PCR-amplified, cloned into the pACT2 vector, and sequenced to confirm that no additional mutations were introduced by the PCR protocol. The death/C-terminal domain (aa 1445–1840) and C-terminal domain (aa 1556–1840) were also cloned into the pAS2-1 vector. Deletions of the C-terminal domain were generated using site-directed mutagenesis (QuikChange XL from Stratagene) converting amino acids Ser1826, Tyr1790, Thr1764, Val1732, Ile1672, and Gln1630 to stop codons. The construct D7 was generated by PCR amplification and cloned into the pAS2-1 vector. MBDR1–24 (aa 31–959), MBDR2–24 (aa 64–959), MBDR3–24 (aa 97–959), MBDR4–24 (aa 130–959), MBDR5–24 (aa 163–959), MBDR6–24 (aa 197–959), MBDR7–24 (aa 227–959), MBDR13–24 (aa 443–959), and MBDR19–24 (aa 628–959) were generated by PCR amplification and cloned into pACT2. Site-directed mutagenesis (Stratagene) was used to mutate amino acids Arg137, Arg40, Arg157, Glu1580, Lys1574, Lys1575, Lys1577/1578, Lys1585/1586, Glu1587, Asp1589, and EED1597/1598/1599 to alanines.

For protein expression in bacteria, the regulatory domain and membrane-binding domain were cloned into the pGex6p-1 vector (Amersham Biosciences). Inserts were PCR-amplified and inserted into pGex6p-1 using EcoR1/XhoI cloning sites. Plasmids encoding eGFP-220-kDa ankyrin-B EED1597AAA and RAAR37AAA were generated by site-directed mutagenesis and subcloned into the parental backbone once the mutated sequence was confirmed.

cDNA of 220-kDa ankyrin-B was subcloned into pBacPak9 using EcoR1/Pmel sites to generate pBacPak9-6His ankyrin-B WT. Subcloning the mutated fragment from pEGFP-ankyrin-B RAAR37AAA using EcoR1/Ndel sites generated the mutant, pBacPak9-6His ankyrin-B RAAR37AAA. Subcloning the mutated region from pEGFP-ankyrin-B EED1597AAA using KpnI/SacII sites generated pBakPak9-6His ankyrin-B EED1597AAA.

Recombinant Protein Expression and Purification—BL21(DE3)-pLysS cells transformed with pGex6p-1 regulatory domain, pGex6p-1 death domain, pGex6p-1 membrane-binding domain, or pGex6p-1 C-terminal domain were grown overnight at 37 °C in LB/amp/chloramphenicol media and sub-cultured the following day for large-scale expression. Cells were grown to an optical density of 0.6–0.8 and induced with 1 mM isopropyl 1-thio-B-D-galactopyranoside for 2 h. Cells were harvested by centrifugation for 10 min at 8,000 × g, resuspended in phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), and frozen at −80 °C following resuspension. Lysis was completed after thawing through activity of lysozyme. The crude extract was syringed through a 22-gauge needle to shear bacterial DNA in lysis buffer (PBS, 1 mM dithiothreitol, 1 mM EDTA, 40 μg/ml 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, 10 μg/ml leupeptin, 40 μg/ml benzamidine, 10 μg/ml pepstatin). Cell debris was removed by centrifugation at 110,000 × g (35,000 rpm) for 1 h. Supernatants were added to 2 ml of equilibrated glutathione-agarose (Amersham Biosciences) batchwise for 4 h at 4 °C. The agarose was washed with 1× PBS containing 0.65 mM NaCl and eluted using elution buffer (50 mM Tris-Cl, 10 mM glutathione, 1 mM EDTA, 1 mM dithiothreitol, pH 8.0). Further purification was performed using high-pressure liquid chromatography on either a Mono Q or Mono S 5/5 HR column. Protein concentrations were determined using Bradford assay. When needed, GST was cleaved using 30 units of Precision Protease (Amersham Biosciences) per milligram of fusion protein and incubated for 6 h at 4 °C. Digests were diazoyed in PBS buffer overnight and re-coupled to glutathione resin for 2 h at 4 °C. All purification steps were analyzed on a Fairbanks SDS-PAGE gradient gel stained with Coomassie Brilliant Blue.

Expression and Purification of Full-length Ankyrin-B—The baculovirus protein expression system was used to express and purify full-length ankyrin-B and mutants. pBacPak9 (Clontech) plasmids encoding ankyrin-B WT, RAAR37AAA, and EED1597AAA were co-transfected into sf21 insect cells along with viral plasmid pBakPak6(Clontech) to generate recombinant virus. Single viral clones were isolated for each ankyrin and amplified for expression. Cells were lysed in 50 mM phosphate buffer, 0.3 mM NaBr, 20 mM imidazole, 0.2 mM β-mercaptoethanol and coupled batchwise to high performance nickel Sepharose (Amersham Biosciences). Sepharose was washed with binding buffer, and proteins were finally eluted in buffer containing 50 mM phosphate buffer, 0.3 mM NaBr, 70 mM imidazole.

In Vitro Protein Interaction—GST-ankyrin-B membrane-binding domain was absorbed to glutathione-Sepharose beads (20 ml of a 10% suspension) and incubated for 2 h at 25 °C in a 100-μl final volume with increasing concentrations of 125I-ankyrin-B CT (63,600 cpm/pmol, labeled with Bolton-Hunter reagent). Samples were layered onto 200 μl of 20% glycerol dissolved in binding assay buffer (20 mM Hepes, pH 7.3, 50 mM NaCl, 1 mM NaEDTA, 0.2% Triton X-100, 1 mM Na2HPO4, 5 mg/ml bovine serum albumin), and beads with absorbed proteins were pelleted by centrifugation at 5,000 × g for 15 min. The tubes were frozen, and tips containing beads were cut off and assayed for 125I in a gamma counter. Values for nonspecific binding were determined using beads with bound GST and were subtracted. The beads were prepared as follows: A 50% slurry of PBS-washed glutathione-Sepharose beads were incubated overnight at 4 °C with either 5 μM purified GST ankyrin-B membrane-binding domain or GST. Beads were then washed with PBS and resuspended in binding assay buffer with the addition of bovine serum albumin.

InsP3 receptor was purified as described previously (15). An InsP3 receptor in vitro competition assay was performed by immobilizing GST-membrane-binding domain on glutathione resin and co-incubating with 1 nm of 125I-labeled InsP3 receptor (specific activity, 4,934,444 cpm/pmol) and increasing concentrations of full-length wild-type ankyrin-B-his, ankyrin-B 1597AAA-his, or ankyrin-B 37AAA-his. Pellets were washed and assayed for 125I using a gamma counter. Values for nonspecific binding were determined by binding 125I-labeled InsP3 receptor to GST-bound glutathione beads and subtracted from total binding.
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RESULTS

Ankyrin-B Regulatory Domain Binds Specifically to the Membrane-binding Domain—220-kDa ankyrin-B has four known folded domains; an 89-kDa membrane-binding domain, a 62-kDa spectrin-binding domain, a 12-kDa death domain, and a 36-kDa C-terminal domain. The combination of death and C-terminal domain is termed the "regulatory domain." B, ankyrin-B regulatory domain interacts with the membrane-binding domain in yeast two-hybrid assays. The ankyrin-B regulatory domain (fused with DNA-binding domain pAS2-1; Bait) was co-transformed into AH109 yeast strain with various ankyrin-B Prey constructs representing various ankyrin-B domains (fused to the DNA activation domain, pACT2). Positive interaction was assessed by growth on media lacking adenine, histidine, leucine, tryptophan (-AHLT). We observed a positive interaction only when pAS2-1 regulatory domain was expressed with pACT2 membrane-binding domain.

Circular Dichroism Spectroscopy—Purified ankyrin-B C-terminal domain and death domain were dialyzed in buffer containing 10 mM Na phosphate, pH 7.4, and 150 mM NaF. 300 μg of a 150 μg/ml solution was loaded onto a 1-mm path-length quartz cuvette. CD measurements were taken on an Aviv 62 DS model CD spectrometer at 25 °C between wavelengths of 260 and 190 nm. Spectra are the averages of five scans. Background signal was determined using buffer alone and subtracted from protein spectra followed by conversion to mean molar ellipticity.

Cardiomyocytes—Neonatal cardiomyocytes were cultured from hearts of P1 mice as described previously (13). For transfection, purified endotoxin-free DNA was isolated from bacteria using Qiagen Midi-preps (Valencia, CA). Eighty-six hours post-isolation, washed cardiomyocytes were transfected using Genefectec reagent (Venn Nova, Pompano, FL) according to the manufacturer’s guidelines (using 2 μg DNA concentration and reagent at 2 mg/ml).

To determine if the ankyrin-B regulatory domain interacts with the membrane-binding domain in solution, we expressed and purified both domains as GST fusion proteins (GST-MBD) in bacteria and immobilized the GST-membrane-binding domain on glutathione resin. The GST tag was cleaved from the regulatory domain using Precision Protease (see "Materials and Methods") for details). B, the approximate stoichiometry of the interaction revealed by the Coomassie-stained gel of bound 125I-labeled RD to GST-MBD after elution from agarose with 5X PAGE sample buffer. The GST-MBD has an approximate molecular mass of 150 kDa, and the regulatory domain migrates nearly twice its predicted molecular mass on SDS-PAGE gels at 82 kDa. C, binding between regulatory and membrane-binding domain is reduced in the presence of higher salt concentrations. Nonspecific binding was evaluated by incubation of GST to 125I regulatory domain.

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FIGURE 1. Identification of an inter-domain interaction within ankyrin-B. A, domain organization of 220-kDa ankyrin-B. Ankyrin-B contains a membrane-binding domain, spectrin-binding domain, death domain, and C-terminal domain. The combination of death and C-terminal domain is termed the "regulatory domain." B, ankyrin-B regulatory domain interacts with the membrane-binding domain in yeast two-hybrid assays. The ankyrin-B regulatory domain (fused with DNA-binding domain pAS2-1; Bait) was co-transformed into AH109 yeast strain with various ankyrin-B Prey constructs representing various ankyrin-B domains (fused to the DNA activation domain, pACT2). Positive interaction was assessed by growth on media lacking adenine, histidine, leucine, tryptophan (-AHLT). We observed a positive interaction only when pAS2-1 regulatory domain was expressed with pACT2 membrane-binding domain.

FIGURE 2. Purified ankyrin-B regulatory domain interacts with purified ankyrin-B membrane-binding domain in solution. A, saturation binding of GST-membrane-binding domain with increasing concentrations of 125I-labeled regulatory domain (RD). Scatchard analysis of saturation binding reveals a Kd of 530 nM (see "Materials and Methods") for details). B, the approximate stoichiometry of the interaction revealed by the Coomassie-stained gel of bound 125I-labeled RD to GST-MBD after elution from agarose with 5X PAGE sample buffer. The GST-MBD has an approximate molecular mass of 150 kDa, and the regulatory domain migrates nearly twice its predicted molecular mass on SDS-PAGE gels at 82 kDa. C, binding between regulatory and membrane-binding domain is reduced in the presence of higher salt concentrations. Nonspecific binding was evaluated by incubation of GST to 125I regulatory domain.
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FIGURE 3. Identification of the ankyrin-B membrane-binding domain site on the ankyrin-B regulatory domain. Yeast AH109 cells were co-transformed with ankyrin-B membrane-binding domain (fused with GAL-4 DNA activation domain) and one of ten prey plasmids containing full-length or partial sequence of the ankyrin-B regulatory domain (amino acids 1445–1840). The death domain does not bind the membrane-binding domain while the C-terminal domain alone maintains binding affinity similar to the regulatory domain construct. The minimal binding region within the C-terminal domain is between amino acids 1556 and 1630. Deletion of this minimal binding region, D7, eliminates the intramolecular interaction.

We further resolved the binding site on the C-terminal domain using deletion mutagenesis (Fig. 3). Truncations were generated by introduction of premature stop codons using site-directed mutagenesis (see “Materials and Methods”). The approach of using deletions is justified, because this domain is highly extended and does not contain significant secondary structure (see Fig. 8 and Table 2). Each C-terminal truncation mutant was co-transformed into yeast with a plasmid containing the ankyrin-B membrane-binding domain in yeast (Fig. 3). In contrast, within the limits of detection, ankyrin-B membrane-binding domain did not interact with the ankyrin-B death domain. Therefore, the ankyrin-B C-terminal domain alone harbors binding sites for the membrane-binding domain (Fig. 3). This finding is consistent with the result that the ankyrin-B death domain does not bind the membrane-binding domain. EED1597 thus represent a set of critical residues in the ankyrin-B C-terminal domain required for the ankyrin-B intramolecular interaction.

Identification of a Membrane-binding Domain Docking Site for the C-terminal Domain—Membrane-binding domain truncation mutants and the yeast two-hybrid system were used to identify the binding site for the ankyrin-B C terminus on the ankyrin-B membrane-binding domain. Membrane-binding domain deletion constructs were designed based upon the boundaries of each ANK repeat (Fig. 5). The first tested deletion construct of the membrane-binding domain encompassed the known binding site for the Insp$_{3}$ receptor (ANK repeats 19–24) (15) (Fig. 5). This construct did not interact with the C-terminal domain by yeast two-hybrid analysis. Additional deletions were generated to encompass ANK repeats 13–24 and 7–24, both of which were also negative for interaction. The remaining ANK repeats were added sequentially to the membrane-binding domain generating the con-
struts MBDR6–24, MBDR5–24, MBDR4–24, MBDR3–24, MBDR2–24, and MBDR1–24 (Fig. 5). Only the construct containing ANK repeats 1–24 showed interaction with the C-terminal domain. Exon one of ankyrin-B encodes a 30-amino acid stretch prior to the first ANK repeat and is not required for binding the C-terminal domain. We conclude that ANK repeat 1 contains a binding site for the C-terminal domain.

ANK repeat does not contain obvious stretches of contiguous charged amino acid likely to interact with the negatively charged C-terminal domain (Fig. 6A). However, the atomic structure for thirteen ankyrin repeats in ankyrin-R reveals that each ANK repeat contains two anti-parallel α helices separated by a short linker (16). We analyzed ANK repeat one using a molecular model of the repeat generated through the three-dimensional model prediction software, Robetta (robetta.bakerlab.org, David Baker). This model reveals the expected anti-parallel α helical structure determined through x-ray crystallography (Fig. 6A). Importantly the model shows the likely position of each amino acid within this repeat structure and reveals that the basic side chains of amino acids Arg37 and Arg40 are positioned together on the same surface of the first helix. We performed site-directed mutagenesis to convert the two residues to alanines and tested association of the full-length membrane-binding domain to the C-terminal domain in yeast two-hybrid analysis (Fig. 6B). We observe that the ankyrin-B membrane-binding domain with amino acid substitutions, Argβ/Argα to Argβ/Argα, is not required for inter-domain interactions.

Amino Acids 1597EED1599 in Ankyrin-B Are Required for Function in Cardiomyocytes—We evaluated the effect of disrupting ankyrin-B inter-domain interactions for ankyrin-B function in vivo using an ankyrin-B−/− cardiomyocyte rescue assay (3, 4, 7, 9). Ankyrin-B is required for normal expression and localization of the InsP3 receptor in ankyrin-B neonatal cardiomyocytes (15). This fact is clearly observed in neonatal cardiomyocytes derived from mice heterozygous for a null mutation in ankyrin-B (ankyrin-B−/− mice), which display reduced expression and abnormal localization of the InsP3 receptor at sites where ankyrin-B is absent (compare Fig. 7, A and B). Abnormal InsP3 receptor phenotypes in ankyrin-B−/− neonatal cardiomyocytes can be rescued to normal by expression of GFP-220-kDa ankyrin-B (13). Moreover, ankyrin-B loss-of-function mutations, including a number of mutants that cause human cardiac arrhythmia, are unable to rescue ankyrin-B−/− phenotypes (3, 4). Therefore, the ankyrin-B−/− cardiomyocyte rescue assay is a valuable system to test ankyrin-B mutants for loss or reduced activity in vivo.

We engineered RAAR37AAA and EED1597AAA mutations into full-length GFP-220-kDa ankyrin-B, and expressed the mutant constructs in ankyrin-B−/− neonatal cardiomyocytes. As previously observed, wild-type GFP-220-kDa ankyrin-B rescued the abnormal expression pattern of InsP3 receptor in ankyrin-B neonatal cardiomyocytes (Fig. 7C). We also observed rescue of InsP3 receptor localization in ankyrin-B−/− neonatal cardiomyocytes transfected with GFP-220-kDa ankyrin-B RAAR37AAA, an ankyrin-B mutant that does not associate with the ankyrin-B C terminus in the yeast two-hybrid assay (Fig. 7D). In striking contrast, mutant GFP-220-kDa ankyrin-B EED1597AAA, in which the C-terminal interaction with the membrane-binding domain is blocked, was ineffective in rescuing InsP3 receptor expression or localization in ankyrin-B−/− neonatal cardiomyocytes. In fact, we observed InsP3 receptor localization in these cells that was even more aberrant than observed in untransfected ankyrin-B−/− neonatal cardiomyocytes. In a small number of transfected cells, we were able to observe a small percentage of InsP3 receptor in a faint striated pattern (see Fig. 7E). However, the majority of ankyrin-B−/− cells transfected with GFP-220-kDa ankyrin-B EED1597AAA displayed a striking perinuclear distribution of InsP3 receptor (Fig. 8, E and F). These data strongly suggest that ankyrin-B inter-domain interactions are critical for ankyrin-B function in vivo. GFP-220-kDa ankyrin-B EED1597AAA and GFP-220-kDa
ankyrin-B RAAR37AAAA are both stably expressed as determined by transfection into 293 cells and immunoblotting using GFP-specific antibodies. The RAAR37AAAA mutation is sufficient to abolish the interaction in the yeast two-hybrid assay, but it is not a loss-of-function mutation. This suggests that the C-terminal domain interacts with one or more additional sites on the membrane-binding domain and/or spectrin-binding domain that are functionally sufficient in vivo. These sites evidently were not detectable in the two-hybrid assay.

**Ankyrin-B Mutants Retain Binding to InsP3 Receptor**—The loss-of-function phenotype associated with the GFP-220-kDa ankyrin-B EED1597AAA construct could result from altered binding to the InsP3 receptor. The ankyrin-B membrane-binding domain alone can bind with high affinity to the InsP3 receptor (15). In a competition assay, ankyrin-B mutants displaced InsP3 receptor binding to the membrane-binding domain with equal activity to wild-type ankyrin-B (Fig. 8). Thus the loss of function observed for the ankyrin-B EED1597AAA-GFP construct is not due to an inability to bind the InsP3 receptor.

**Structural Properties of the C-terminal Domain**—We performed a detailed structural analysis of the ankyrin-B C-terminal domain to provide insight into its folding properties. The C-terminal domain contains an unusually high degree of charged and polar amino acids suggesting that this domain may not form a globular structure in solution. Consistent with this prediction, the CD profile of the C-terminal domain revealed predominantly random coil structure. Specifically, we observed a single minimum at 200 nm and a slightly downward inflection at 222 nm representing a maximum of 10%/H9851 helical content (Fig. 9A). Circular dichroism analysis for the ankyrin-B death domain revealed an /H9851 helical content of 60% (Fig. 9B) confirming the accuracy of this assay for determining secondary structure content (e.g., death domains typically contain from 53–73%/H9851 helix as determined by x-ray crystallography (14)).

Physical properties of the C-terminal domain were further analyzed through the determination of a Stokes radius and sedimentation coefficient (17). Our analysis revealed a Stokes radius of 6.1 nm and a sedimentation coefficient of 1.6 s. These values indicate that the C-terminal domain is a highly extended monomer in solution with a calculated frictional ratio of 2.44 and a molecular mass of 35,743 Da (Table 1). For comparison, spectrin, which has an elongated flexible shape, has a frictional ratio of 2.9 (12). The C-terminal domain thus is comparable to spectrin in shape, but may be more flexible.

**Physical Properties of Wild-type and Mutant Ankyrin-B**—To determine the structural consequences of eliminating the inter-domain interaction we determined a Stokes radius and sedimentation coefficient (17). Our analysis revealed a Stokes radius of 6.1 nm and a sedimentation coefficient of 1.6 s. These values indicate that the C-terminal domain is a highly extended monomer in solution with a calculated frictional ratio of 2.44 and a molecular mass of 35,743 Da (Table 1). For comparison, spectrin, which has an elongated flexible shape, has a frictional ratio of 2.9 (12). The C-terminal domain thus is comparable to spectrin in shape, but may be more flexible.
revealed that ankyrin-B wild-type and ankyrin-B RAAR^{37}AAAA each have sedimentation coefficient values of 6.7 s. In contrast, ankyrin-B EED^{1597}AAA has a sedimentation coefficient value of 6.3 s (Table 2). We next determined the Stokes radius value for each ankyrin by gel filtration. The Stokes radius for wild-type ankyrin-B and ankyrin-B RAAR^{37}AAAA was 7.8 nm. In contrast, the Stokes radius of ankyrin-B EED^{1597}AAA is 8.2 nm (Table 2). Ankyrin-B EED^{1597}AAA has a smaller sedimentation coefficient combined with larger Stokes radius, indicating a more extended shape in solution. This is consistent with the interpretation that loss of intramolecular interactions leads to an extended conformation of ankyrin-B.

Based on these values for Stokes radius and sedimentation coefficient, the calculated molecular masses in solution of wild-type ankyrin-B, ankyrin-B RAAR^{37}AAAA, and ankyrin-B EED^{1597}AAA are 208,912, 208,912, and 206,514 Da, respectively (Table 2). Wild-type ankyrin-B and mutants therefore are monomers and do not self-associate at least in relatively dilute solution. Thus the inter-domain interaction occurs through intra-molecular interactions rather than through inter-molecular interactions between ankyrin-B polypeptides.

**DISCUSSION**

We establish in this report a direct interaction between the membrane-binding domain and the C-terminal domain in ankyrin-B using purified proteins in solution and the yeast two-hybrid assay. Amino acids^{1597}EED^{1599} in ankyrin-B are identified here to be both critical for the intramolecular interaction as well as ankyrin-B function in cardiomyocytes. Circular dichroism analysis and hydrodynamic parameters reveal that the C-terminal domain is a random coil in solution. Structural analysis of full length ankyrin-B and mutants shows that conversion of amino acids^{1597}EED^{1599} to alanine leads to a conformational change in ankyrin-B leading to a more extended shape. These observations suggest that the C-terminal domain in ankyrin-B folds back to interact with the membrane-binding domain and that release of this interaction leads to extension of the ankyrin-B protein. They also suggest the use of intra-molecular interactions as a mechanism for the regulation of ankyrin-B function in vivo.

The^{1597}EED^{1599} mutations that block intramolecular interaction between the C-terminal and membrane-binding domains also eliminate activity of ankyrin-B in cardiomyocytes. However, this mutation does not alter affinity of ankyrin-B for the InsP3 receptor. As speculation, the C-terminal domain may promote conformational changes that are required for functional activity in vivo. Additionally the C-terminal domain may modulate binding to unidentified regulatory partners required for ankyrin-B activity.

C-terminal domains in ankyrins are the most divergent among ankyrin-folded regions. The intramolecular interaction site on the ankyrin-B C-terminal domain maps to the N terminus of the C-terminal domain. When aligning the C-terminal domain between ankyrins B and G we observe an insertion in the alignment for the ankyrin-B C-terminal domain at its N terminus and thus the largest non-homologous segment between these divergent domains (Fig. 10). This region of the ankyrin-B C-terminal domain includes the important^{1597}EED^{1599} sequence required for intramolecular interaction.

Ankyrin-B isoforms also contain inserted sequences outside their C-terminal domains. The neuronal-specific isoform, 440-kDa ankyrin-B, contains an inserted 2085-amino acid stretch after the spectrin-binding domain and before the death domain (19). This large insertion places the membrane-binding domain and C-terminal domain considerably further apart from one another in the linear sequence and may eliminate any potential intramolecular interaction between these two domains. Ankyrin-G isoforms with alternative C-terminal domains have been

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**TABLE 2**

**Structural properties of ankyrin-B**

| Properties | Values |
|------------|--------|
| Sedimentation coefficient, $s_{20,w}$ | 6.7 s (WT) / 6.3 s (EED^{1597}AAA) / 6.7 s (RAAR^{37}AAAA) |
| Partial specific volume, $\psi$ | 0.713 cm$^3$/g (WT) / 0.713 cm$^3$/g (EED^{1597}AAA) / 0.713 cm$^3$/g (RAAR^{37}AAAA) |
| Stokes radius, $R_s$ | 7.8 nm (WT) / 8.2 nm (EED^{1597}AAA) / 7.8 nm (RAAR^{37}AAAA) |
| Molecular weight, calculated | 208,912 (WT) / 206,514 (EED^{1597}AAA) / 208,912 (RAAR^{37}AAAA) |
| Molecular weight, actual | 202,393 (WT) / 202,233 (EED^{1597}AAA) / 202,223 (RAAR^{37}AAAA) |
| Frictional ratio, $f/f_0$ | 1.78 (WT) / 1.88 (EED^{1597}AAA) / 1.78 (RAAR^{37}AAAA) |

*From sucrose gradient sedimentation.

*Estimated from the amino acid composition.

*Calculated from the equation, $s_{20,w} = (6\pi NR^2 v_0)/((1 - v_p/v))^{1/3}$ (Eq. 1).

*Calculated from gel filtration on a calibrated Superose 6 column.

*Calculated from the amino acid sequence.

*Calculated from gel filtration on a calibrated Superose 12 column.

*From sucrose gradient sedimentation.

*Estimated from the amino acid composition.
The usefulness of this type of protein structure is in the types of protein-protein interactions that derive from it. These interactions would be of global structure and membrane-binding domain as their mechanism for driving activity and/or specificity.

The highly extended nature of the C-terminal domain provides insight into how this interaction may look if one had a crystal structure of ankyrin-B. We would envision that the C-terminal domain would overlap the membrane-binding domain at multiple contact sites due to its extended nature. This is evident by the fact that conversion of amino acids Arg37 and Arg40 were sufficient to eliminate interaction in the yeast two-hybrid but did not lead to a change in conformation or a loss of function suggesting the presence of additional contact sites for the C-terminal domain. The interaction may have similarity to the Ezrin, Radixin, Moesin (ERM) family of proteins in which the protein structure has revealed considerable overlap of interaction of the C-terminal tail domain with the N-terminal FERM domain (21).

The ankyrin-B C-terminal domain falls into a class of intrinsically unstructured proteins. These proteins are categorized on three basic properties (22). The first is an unusually high composition of polar and charged amino acids, which would drive an extended rather than a global structure in vivo. The second is a circular dichroism profile that contains a large random coil minimum at 200 nm with little to no secondary structure. Finally these proteins tend to migrate slower than predicted from their molecular weight on SDS-PAGE gels. The ankyrin-B C-terminal domain meets all of these characteristics and should be a new addition to this class of proteins. Another interesting characteristic of this class of proteins is that many can gain secondary structure upon binding their respective partners. Thus many ankyrin-binding proteins in addition to the C-terminal have adopted unstructured regions in their polypeptides. An anion exchanger cytoplasmic domain was recently found to be required for association to ankyrin-B by x-ray crystallography (23). Neurofascin and other members of the L1 CAM family also associate with ankyrins through highly extended and unstructured cytoplasmic domains (24). In addition, phosphorylation at a FIIQY motif in the L1 CAM C-terminal domain leads to a loss of interaction with ankyrin-G revealing that this type of interaction can be regulated through kinase activity (25). Ankyrins bind to integral membrane proteins predominately through ankyrin repeats, which fold to form a large super-helical structure generating a considerably large surface area for which protein-protein interaction can occur (16). Thus many ankyrin-binding proteins in addition to the C-terminal have adopted unstructured and extended domains to associate with this unique ankyrin repeat superhelical structure.

We present in this study the first observation of an intramolecular interaction in ankyrin-B. The interaction between C-terminal domain and membrane-binding domain of ankyrin-B represents a mechanism for regulating ankyrin-B function. The intramolecular interaction itself could be regulated through other regulatory proteins, phosphorylation, or proteolytic activity. Amino acids near 1597EED1599 and 37RAAR40 in ankyrin-B represent potential locations for phosphorylation sites.

3. K. M. Abdi, P. J. Mohler, J. Q. Davis, and Vann Bennett, unpublished data.
Intramolecular Associations in Ankyrin-B

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