Ankyrin-B Targets \( \beta_2 \)-Spectrin to an Intracellular Compartment in Neonatal Cardiomyocytes*§

Peter J. Mohler‡§∥, Woohyun Yoon‡∥, and Vann Bennett‡§∥**

From the ¶Howard Hughes Medical Institute and Departments of §Cell Biology, ¶Biochemistry, and **Neurosciences, Duke University Medical Center, Durham, North Carolina 27710

Ankyrin-B is a spectrin-binding protein that is required for localization of inositol 1,4,5-trisphosphate receptor and ryanodine receptor in neonatal cardiomyocytes. This work addresses the interaction between ankyrin-B and \( \beta_2 \)-spectrin in these cells. Ankyrin-B and \( \beta_2 \)-spectrin are colocalized in an intracellular striated compartment overlying the M-line and distinct from T-tubules, sarcoplasmic reticulum, Golgi, endoplasmic reticulum, lysosomes, and endosomes. \( \beta_2 \)-Spectrin is absent in ankyrin-B-null cardiomyocytes and is restored to a normal striated pattern by rescue with green fluorescent protein-220-kDa ankyrin-B. We identified two mutants (A1000P and DAR976AAA) located in the ZU5 domain which eliminate spectrin binding activity of ankyrin-B. Ankyrin-B mutants lacking spectrin binding activity are normally targeted but do not reestablish \( \beta_2 \)-spectrin in ankyrin-B-deficient cardiomyocytes. However, both mutant forms of ankyrin-B are still capable of restoring inositol 1,4,5-trisphosphate receptor localization and normal contraction frequency of cardiomyocytes. Therefore, direct binding of \( \beta_2 \)-spectrin to ankyrin-B is required for the normal targeting of \( \beta_2 \)-spectrin in neonatal cardiomyocytes. In contrast, ankyrin-B localization and function are independent of \( \beta_2 \)-spectrin. In summary, this work demonstrates that interaction between members of the ankyrin and \( \beta_2 \)-spectrin families previously established in erythrocytes and axon initial segments also occurs in neonatal cardiomyocytes with ankyrin-B and \( \beta_2 \)-spectrin. This work also establishes a functional hierarchy in which ankyrin-B determines the localization of \( \beta_2 \)-spectrin and operates independently of \( \beta_2 \)-spectrin in its role in organizing membrane-spanning proteins.

Ankyrins are a closely related family of membrane adaptors required for organizing diverse membrane-spanning proteins including ion channels and transporters and L1 CAM cell adhesion molecules in physiologically important membrane domains (1, 2). Targeted knock-out of ankyrin-G in the mouse cerebellum results in loss of clustering of the voltage-gated sodium channel Na\(_1\),6 and L1 CAMs neurofascin and NrCAM at Purkinje neuron initial segments (3, 4). Knock-down of ankyrin-G in cultured epithelial cells by small interfering RNA results in loss of the lateral membrane domain (5). Loss of ankyrin-B in mice results in deficiency of Na,K-ATPase, Na/Ca exchanger, and inositol 1,4,5-trisphosphate (InsP\(_3\))\(^1\) receptor located in a specialized microdomain of T-tubules in adult cardiomyocytes (6). Humans with loss-of-function mutations in ankyrin-B and mice heterozygous for a null mutation in ankyrin-B (ankyrin-B\(^{-/}\) mice) display stress-induced cardiac arrhythmia and sudden cardiac death (6, 7).

The identity of cellular pathway(s) involved in ankyrin-dependent organization of membrane-spanning proteins is an important and currently unresolved question. Clues may come from elucidating proteins that interact with ankyrins. Erythrocyte spectrin, which is assembled with actin in a two-dimensional network, was the first ankyrin-binding protein to be identified. Ankyrin was in fact discovered based on its role as the membrane attachment site for spectrin in erythrocyte membranes (8–11). Mutations of erythrocyte ankyrin result in deficiency of spectrin and cause hereditary spherocytosis in humans and mice (12–14). Members of the spectrin family also collaborate with ankyrinB in cells other than erythrocytes. \( \beta_2 \)-Spectrin colocalizes with ankyrin-G at axon initial segments and is lost in ankyrin-G knock-out mice (4). Conversely, ankyrin-G exhibits reduced levels at axon initial segments and nodes of Ranvier of \( \beta_2 \)-spectrin mutant mice (15).

This work addresses the interaction between ankyrin-B and \( \beta_2 \)-spectrin in neonatal cardiomyocytes. Ankyrin-B is required for localization of InsP\(_3\) receptor and ryanodine receptor in the sarcoplasmic reticulum of these cells based on studies with primary cultures from ankyrin-B mutant mice (6, 7, 16–18). Ankyrin-B contains a 62-kDa spectrin binding domain that associates with high affinity (\( K_d \sim 25 \) nM) with \( \beta_2 \)-spectrin (19). \( \beta_2 \)-Spectrin spliceoforms are expressed in adult heart (20), although their localization with respect to ankyrin-B has not been evaluated. Here we report that \( \beta_2 \)-spectrin is a physiological binding partner for ankyrin-B in neonatal cardiomyocytes. We also provide evidence for a functional hierarchy in which ankyrin-B determines the localization of \( \beta_2 \)-spectrin and operates independently of \( \beta_2 \)-spectrin in its role in organizing membrane-spanning proteins.

**EXPERIMENTAL PROCEDURES**

**Immunoblot Analysis**—Quantitative Western blot analysis was performed using affinity-purified antibodies, \(^{125}\)I-labeled protein A, and phosphorimaging (17).

**Yeast Two-hybrid Assays**—The yeast two-hybrid system was used to assay the interaction between ankyrin-B and \( \beta_2 \)-spectrin. Full-length and truncated fragments of the 220-kDa ankyrin-B spectrin binding domain were PCR amplified and inserted into pAS2-1 (Clontech). The

* This work was supported by the Howard Hughes Medical Institute and Johnson and Johnson. 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.

§ The on-line version of this article (available at http://www.jbc.org) contains Supplemental Fig. 1.

∥ To whom correspondence should be addressed: Dept. of Cell Biology, Howard Hughes Medical Institute, Box 3892, Duke University Medical Center, Durham, NC 27710. Tel.: 919-684-3027; Fax:919-684-3590; E-mail: p.mohler@cellbio.duke.edu or v.bennett@cellbio.duke.edu.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
known ankyrin binding domain of β2-spectrin was PCR amplified and inserted into pACT2. All constructs were completely sequenced and were free of mutations. Fusion protein expression in yeast was confirmed by Western blot analyses using commercially available binding domain and activation domain antibodies (Clontech). Plasmids were cotransformed into yeast (AH109) and assayed using ADE2, HIS3, and lacZ selection (Clontech). Transformers that exhibited growth on −Ade/His/Leu/Trp media were considered positive for interaction.

**Ankyrin-B Mutagenesis**—Deletion constructs of human ankyrin-B spectrin binding domain were generated by PCR. Spectrin binding domain constructs correspond to residues 862–953 (D1), 862–1070 (D2), 862–1125 (D3), 862–1319 (D4), 986–1443 (D5), 1071–1443 (D6), 1158–1443 (D7), 966–1125 (D8), and 966–1125 (D9) of human 220-kDa ankyrin-B. These constructs were ligated into pAS2-1 and sequenced to verify that there were no mutations introduced by PCR. Alanine mutations were introduced into the D9 deletion construct (residues 966–1125) of 220-kDa ankyrin-B spectrin binding domain using the QuickChange XL Site-directed Mutagenesis Kit (Stratagene). A total of 862–1125 (D1), 966–1125 (D2), and 1071–1443 (D6) were subcloned back into the bait plasmid, and loss-of-interaction was confirmed by PCR and sequencing. Clones with only one mutation were selected for high probability of location on the surface of the domain; second, use random mutagenesis to create loss-of-binding mutations. Both approaches utilized the yeast two-hybrid assay. We constructed full-length and truncated polypeptides of the ankyrin-B spectrin binding domain library created using GeneMorph PCR mutagenesis (Stratagene). The PCR protocol was optimized to obtain mutation frequency of 1 nucleotide mutation/ankyrin-B spectrin binding domain (∼1.75 kb). Mutagenized products were cloned into pAS2-1 vector, and clones were amplified to create a mutant library for yeast two-hybrid screening. Clones were cotransformed with mutant library and pACT2 β2-spectrin ankyrin binding domain (residues 1563–2093) and plated on −LT media. Single colonies were replicated on −ALT and −AHLT plates to identify cotransformants that did not interact with β2-spectrin. Noninteracting ankyrin-B clones were analyzed by immunoblot to confirm absence of stop codons, and positive clones were then analyzed for their expression levels by PCR and sequencing. Clones with only one mutation were subcloned back into the bait plasmid, and loss-of-interaction was confirmed.

**Antibodies**—Antibodies include affinity-purified antibodies to β2-spectrin (21), GFF (17), InAr (receptors) (17), and ankyrin-B (17) as well as commercial antibodies to SERCA2, β-COP, ryanodine receptor (ABR), α-actinin (Sigma), Golgin (Molecular Probes), EEA1, BIP (GRP78) (Transduction), and LAM1 (Santa Cruz).

**Immunofluorescence**—Neonatal cardiomyocytes were washed with phosphate-buffered saline, pH 7.4, and fixed in warm 4% paraformaldehyde (37 °C). Cells were blocked/permeabilized in phosphate-buffered saline containing 0.075% Triton X-100 and 3% fish oil gelatin (Sigma) and incubated with primary antibody overnight at 4 °C. After washes (phosphate-buffered saline plus 0.1% Triton X-100), cells were incubated in secondary antibody (Alexa 488, 568, 633; Molecular Probes) for 8 h at 4 °C and mounted using Vectashield (Vector) and no. 1 coverslips. Images were collected on Zeiss 510 Meta confocal microscope (100 power oil 1.45 NA (Zeiss), pinhole equals 1.0 Airy Disc) using Carl Zeiss Imaging software. All channels were collected on PMT3. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment. Three-dimensional images were created using 0.18-μm-thick Z-scans and assembled using Velocity Software (Improvision).

**Neonatal Cardiomyocytes**—Neonatal cardiomyocytes were isolated from P1-P2 mice and transfected as described previously (16). After transfection, isolated cardiomyocyte spontaneous contraction rates and/or Ca2+ dynamics (Fluo-3 AM) were monitored as described previously (6).

**Statistics**—When appropriate, data were analyzed using a two-tailed Student’s t test, and values less than p < 0.05 were considered significant. Values are expressed as the mean ± S.D.

**RESULTS**

**Identification of Mutations Eliminating Spectrin Binding Activity of Ankyrin-B**—We needed ankyrin-B mutants lacking spectrin binding activity to evaluate the potential role of β2-spectrin in cellular localization and function of ankyrin-B (see below). Two strategies were employed to accomplish this goal: first, identify the minimal spectrin binding domain and then evaluate alanine-scanning mutations of surface residues within this domain; second, use random mutagenesis to create loss-of-binding mutations. Both approaches utilized the yeast two-hybrid assay. We constructed full-length and truncated polypeptides of the ankyrin-B spectrin binding domain (residues 862–1443) fused to the GALA DNA binding domain (see Fig. 1B). We also fused the minimal ankyrin binding domain of β2-spectrin (repeats 13–17, residues 1563–2093 (23)) with the GAL4 activation domain. As expected, the 62-kDa ankyrin-B domain interacts with β2-spectrin residues 1563–2093 in yeast (Fig. 1B). Truncated ankyrin-B constructs were designed based on the previous finding that limited proteolysis of the spectrin binding domain of ankyrin-R yields a subdomain corresponding to the ZU5 domain plus an additional C-terminal sequence (24). ZU5 domains are a feature shared with the proteins ZO-1 and Unc5 (smart.embl-heidelberg.de). The predicted ZU5 domain did not have β2-spectrin binding activity. However, the 55 residues C-terminal to the predicted ZU5 domain have homology with a stretch of Unc5 but are missing in ZO-1 (not shown). The predicted ZU5 domain plus 55 C-terminal residues had β2-spectrin binding activity equivalent to full-length constructs, whereas constructs N-terminal and C-terminal to this stretch were inactive (Fig. 1B). These results define a 160-amino acid β2-spectrin binding sequence in the 62-kDa ankyrin-B domain.

Alanine-scanning mutagenesis of predicted surface residues on the minimal spectrin binding domain (Fig. 1C, construct D9, amino acids 966–1125) was performed to identify mutants lacking spectrin binding activity. A total of 13 sets of residues selected for high probability of location on the surface of the folded domain (i.e. 2 or more consecutive charged amino acids) were changed to alanines (two to four alanine substitutions/site; Fig. 1C). These mutants were cotransformed with β2-spectrin in yeast. 11 of the 13 mutants did not affect the ankyrin-B/β2-spectrin interaction (Fig. 1C). However, two alanine mutants affected ankyrin-B interaction with β2-spectrin. One mutant, DAR976AAA, completely abolished interaction with β2-spectrin, whereas the second mutant, ENGD1070AAA, reduced but did not eliminate the ankyrin-B/β2-spectrin interaction (we observed minimal growth on AHLT medium; Fig. 1C).

We also used random mutagenesis with yeast two-hybrid analysis to identify additional loss-of-binding mutations and to confirm independently the minimal spectrin binding domain. A mutant ankyrin-B spectrin binding domain library was created using PCR mutagenesis (see “Experimental Procedures”). Yeast cells were cotransformed with the mutagenized library and β2-spectrin ankyrin binding domain (residues 1563–2093). Ankyrin-B mutants that did not interact with β2-spectrin and produced full-length ankyrin-B spectrin binding domain by immunoblot analysis were sequenced completely. Clones with only one mutation were subcloned back into the bait plasmid, and loss-of-interaction was confirmed. A screen of ~4,000 clones identified one that did not interact with β2-spectrin. This mutant contained a single base mutation (G2998C) resulting in an alannine-to-proline substitution at amino acid 1000 (A1000P; Fig. 1B). A1000P is located within the minimal spectrin binding construct identified by truncation analysis (Fig. 1B) and is very close to the DAR976AAA loss-of-binding mutant.

These experiments identified two mutations that eliminate spectrin binding activity of ankyrin-B and established a domain encompassing the predicted ZU5 domain as the minimal
The minimal analysis of ankyrin-B and expression in heart was determined by quantitative immunoblot analysis of ankyrin-B and spectrin binding activity of other ankyrins. The ZU5 domain in ankyrin-B residues 968–1125 (construct D9). The predicted ZU5 domain in ankyrin-B (D8, residues 966–1070) did not interact with β2-spectrin, WT, wild-type; C, predicted surface residues of the minimal β2-spectrin binding sequence were mutated to alanines and tested for β2-spectrin binding. The Ala to Pro mutation (A1000P) was identified by random mutagenesis and the DAR976AAA mutation from alanine mutagenesis both blocked β2-spectrin binding. The ENGD108TAAAGA mutant showed reduced growth rate of yeast on AHLT selection consistent with a reduced affinity for β2-spectrin. D, conserved sequence of the N terminus of the ankyrin-B β2-spectrin binding site. DAR residues (boxed) critical for spectrin binding are conserved in ankyrins throughout evolution (hAnkR, human ankyrin-B; hAnkG, human ankyrin-G; hAnkR, human ankyrin-B; Unc-44, C. elegans ankyrin; dAnk1, Drosophila ankyrin 1). Human Unc-5 (hUnc-5) contains a ZU5 domain but does not interact with β2-spectrin.

Ankyrin-B Targets β2-Spectrin in Neonatal Cardiomyocytes

Fig. 1. Identification of single amino acid mutations that eliminate 220-kDa ankyrin-B spectrin binding activity. A, domain organization of 220-kDa ankyrin-B including membrane binding domain (MBD), spectrin binding domain (SBD), death domain (DD), and C-terminal domain (CTD). The blue hatched box in the spectrin binding domain represents a predicted ZU5 domain (residues 966–1070). B, 220-kDa ankyrin-B spectrin binding domain truncation constructs D1–D9 were tested for ability to bind β2-spectrin by yeast two-hybrid analysis. Positive interaction with β2-spectrin is displayed on the AHLT selection plate (right). The minimal β2-spectrin binding domain includes ankyrin-B residues 968–1125 (construct D9). The predicted ZU5 domain in ankyrin-B (D8, residues 966–1070) did not interact with β2-spectrin. WT, wild-type; C, predicted surface residues of the minimal β2-spectrin binding sequence were mutated to alanines and tested for β2-spectrin binding. The Ala to Pro mutation (A1000P) was identified by random mutagenesis and the DAR976AAA mutation from alanine mutagenesis both blocked β2-spectrin binding. The ENGD108TAAAGA mutant showed reduced growth rate of yeast on AHLT selection consistent with a reduced affinity for β2-spectrin. D, conserved sequence of the N terminus of the ankyrin-B β2-spectrin binding site. DAR residues (boxed) critical for spectrin binding are conserved in ankyrins throughout evolution (hAnkR, human ankyrin-B; hAnkG, human ankyrin-G; hAnkR, human ankyrin-B; Unc-44, C. elegans ankyrin; dAnk1, Drosophila ankyrin 1). Human Unc-5 (hUnc-5) contains a ZU5 domain but does not interact with β2-spectrin.

Reduction in Ankyrin-B Expression in Neonatal Cardiomyocytes

Results in Abnormal Targeting and Expression of β2-Spectrin—we determined the predominant β2-spectrin isoform(s) present in mouse neonatal cardiomyocytes using an affinity-purified β2-spectrin antibody raised against spectrin repeats 4–9 (21). This β2-spectrin antibody does not cross-react with β2-spectrin from red blood cell ghosts but does recognize a 274-kDa β2-spectrin isoform in whole brain lysates (Fig. 2A). Immunoblot analysis of wild-type neonatal mouse cardiomyocytes lysates revealed that 274-kDa β2-spectrin is the major isoform in developing ventricular cardiomyocytes (Fig. 2B). We did not observe lower molecular mass β2-spectrin isoforms in neonatal cardiomyocytes, including 240- or 180-kDa β2-spectrin isoforms observed in adult heart (data not shown).

The potential requirement of ankyrin-B for β2-spectrin expression in heart was determined by quantitative immunoblot analysis of ankyrin-B and β2-spectrin in wild-type and ankyrin-B−/− neonatal cardiomyocytes. Consistent with results from adult ankyrin-B−/− heart and isolated cardiomyocytes (6), ankyrin-B−/− neonatal cardiomyocytes display nearly a 50% reduction in expression of 220-kDa ankyrin-B (Fig. 2, B and C, n = 4, p < 0.05 (6)). Expression levels of the predominant β2-spectrin isoform (274-kDa β2-spectrin) were also decreased >35% in ankyrin-B−/− neonatal cardiomyocytes (Fig. 2, B and C, n = 4, p < 0.05). There was no difference in expression levels of other cardiomyocyte structural components including α-actinin (Fig. 2C; n = 4, p < 0.05). Ankyrin-B-null neonatal cardiomyocytes were not examined by immunoblot because of the scarcity of null animals, which die soon after birth.

β2-Spectrin staining by immunofluorescence is nearly eliminated in ankyrin-B-null cardiomyocytes (the cell in this example is a cardiomyocyte as evidenced by α-actinin staining; Fig. 2E). Ankyrin-B in ankyrin-B−/− neonatal cardiomyocytes is localized in a chimeric pattern where distinct regions of the cell display nearly normal ankyrin-B localization, and other regions of the cell are nearly completely lacking ankyrin-B staining (Fig. 1F). β2-Spectrin expression in ankyrin-B−/− precisely mimics the subcellular chimeric distribution of ankyrin-B and is missing from the same areas of these cells lacking ankyrin-B (Fig. 1F). Loss of ankyrin-B in either ankyrin-B−/− or ankyrin-B−/− cardiomyocytes does not affect the localization of other cardiomyocyte proteins marking the contractile apparatus (α-actinin), endoplasmic reticulum/sarcoplasmic reticulum (sarco-
plasmic reticulum calcium-ATPase 2 (SERCA2)), or nascent T-tubules (dihydropyridine receptor) (18).

We next tested whether abnormal localization of β2-spectrin in ankyrin-B−/− neonatal cardiomyocytes could be restored to normal by expression of exogenous GFP-ankyrin-B. Reduction of ankyrin-B in ankyrin-B−/− and ankyrin-B−/− neonatal cardiomyocytes leads to abnormal cardiomyocyte spontaneous contraction rates and abnormal localization and expression of ankyrin-B-associated proteins including InsP3 receptor, ryanodine receptor, Na,K-ATPase, and Na/Ca exchanger (6, 17, 18). These abnormal phenotypes can be rescued by transfection of the cardiomyocytes with GFP-220-kDa ankyrin-B (6, 17, 18).

We expressed GFP-220-kDa ankyrin-B in 3-day-old ankyrin-B−/− neonatal cardiomyocytes and 36 h later determined the localization of GFP-220-kDa ankyrin-B using an affinity-purified GFP antibody. GFP-220-kDa ankyrin-B was localized in a striated pattern similar to that of endogenous ankyrin-B (Fig. 3B). Transfection of GFP-220-kDa ankyrin-B rescues the expression and localization of β2-spectrin in neonatal cardiomyocytes (Fig. 3B). These results demonstrate that ankyrin-B is required for β2-spectrin targeting and expression in neonatal cardiomyocytes.

Direct Interaction with Ankyrin-B Is Required for β2-Spectrin Targeting in Neonatal Cardiomyocytes—We used the ankyrin-B−/− cardiomyocyte rescue assay and ankyrin-B mutants lacking β2-spectrin binding to determine whether direct interaction between ankyrin-B and β2-spectrin is required for β2-spectrin targeting in neonatal cardiomyocytes. GFP-ankyrin-B EDE1082AAA, an ankyrin-B mutant in the spectrin binding region which does not affect binding to β2-spectrin (Fig. 1C), is localized as wild-type GFP-220-kDa ankyrin-B and also rescues β2-spectrin localization in ankyrin-B−/− neonatal cardiomyocytes (Fig. 4B). In contrast, GFP-220-kDa ankyrin-B mutants that lack β2-spectrin binding (DAR976AAA and A1000P) did not rescue the localization of β2-spectrin, even though they were normally expressed and correctly targeted in ankyrin-B−/− neonatal cardiomyocytes (Fig. 4, C and D). Therefore, direct binding of β2-spectrin to ankyrin-B is required for the normal targeting of β2-spectrin in neonatal cardiomyocytes. Moreover, localization of ankyrin-B in cardiomyocytes occurs independently of β2-spectrin.

Ankyrin-B-dependent Targeting of the InsP3 Receptor Is Independent of β2-Spectrin Activity—The role of β2-spectrin in ankyrin-B-dependent targeting of the InsP3 receptor was determined by rescue experiments using GFP-220-kDa ankyrin-B mutants lacking β2-spectrin binding activity. Abnor-
ankyrin-B GFP-220-kDa ankyrin-B EDE1082AAA was unable to rescueous contraction rates to normal levels (Fig. 6). In contrast,ankyrin-B and have full activity in rescuing InsP3 receptor localization.

length ankyrin-B because these mutants are normally targetedA1000P nor DAR976AAA mutations affect the folding of full-
have full activity (Fig. 1C) and rescues localization of
this mutant maintained normalmal localization and expression of InsP3 receptor in ankyrin-B
—
even though this mutant has full 2-spectrin binding activity (Fig. 1C) and rescues localization of
2-spectrin requires di-
2-spectrin do not bind 2-spectrin localization in ank
dard cardiomyocytes display reduced spontaneous contraction rates and abnormal Ca2+
contraction rates (18) was assessed using ankyrin-B DAR976AAA and A1000P mutants. Compared with wild-type neonatal cardiomyocytes, ank
ankyrin-B 220-kDa ankyrin-B A1000P (both lack 2-spectrin binding activity) does not rescue abnormal 2-spectrin targeting in ank
220-kDa ankyrin-B DAR976AAA (C) or A1000P (D) (both lack 2-
spectrin binding activity) does not rescue abnormal 2-spectrin targeting in ank
ankyn-B 220-kDa ankyrin-B EDE1082AAA has full 2-
spectin binding activity and rescues 2-
spectrin localization in ank
—
ankyn-B with 2-spectrin is not required for ank
—
ankyn-B 220-kDa ankyrin-B rescues abnormal localization of 2-
spectrin in ank
2-Spectrin are Colocalized in an Intracellular Striated Compartment Distinct from Known Membrane Structures—We compared the localization of ank
—
—
A1000P or DAR976AAA restored ank
—
ankyn-B 220-kDa ankyrin-B EDE1082AAA was unable to rescue ank
—
ankyn-B 220-kDa ankyrin-B A1000P or DAR976AAA restored ank
—
2-spectrin activity in ank
—
neonatal cardiomyocytes immu-
Experimental Procedures

Fig. 5. Ankyrin-B/β₂-spectrin interaction is not required for ankyrin-B-dependent targeting of the InsP₃ receptor. A and B, localization of ankyrin-B and InsP₃ receptor (IP3R) in wild-type and ankyrin-B⁻/⁻ neonatal cardiomyocytes. C-F, localization of WT and mutant GFP-ankyrin-B and β₂-spectrin in transfected ankyrin-B⁻/⁻ neonatal cardiomyocytes. The scale bar equals 10 μm. Note that InsP₃ receptor localization is rescued by two GFP-220-kDa ankyrin-B mutants that do not associate with β₂-spectrin. Ankyrin-B⁻/⁻ cardiomyocytes expressing GFP-220-kDa ankyrin-B EDE1082AAA at similar levels display abnormal localization and expression of InsP₃ receptors (small puncta throughout the cell).

Fig. 6. Ankyrin-B/β₂-spectrin interaction is not required for ankyrin-B-dependent rescue of neonatal cardiomyocyte contractility. Spontaneous contraction rates recorded from wild-type and ankyrin-B⁻/⁻ neonatal cardiomyocytes and ankyrin-B⁻/⁻ neonatal cardiomyocytes transfected with GFP-220-kDa ankyrin-B, GFP-220-kDa ankyrin-B, GFP-220-kDa ankyrin-B A1000P, GFP-220-kDa ankyrin-B DAR976AAA, GFP-220-kDa ankyrin-B EDE1082AAA, and GFP. Ankyrin-B mutants that blocked β₂-spectrin binding did not affect rescue of cardiomyocyte spontaneous contraction rates. Data represent three experiments from three mice (p < 0.05).

Ankyrin-B Targets β₂-Spectrin in Neonatal Cardiomyocytes

DISCUSSION

This work demonstrates that ankyrin-B is required for localization of β₂-spectrin to an intracellular compartment in neonatal cardiomyocytes. β₂-Spectrin is colocalized with ankyrin-B in a striated pattern overlaying the M-line. β₂-Spectrin is absent in ankyrin-B-null cardiomyocytes and is restored to a normal striated pattern by rescue with GFP-220-kDa ankyrin-B. Ankyrin-B mutants lacking spectrin binding activity do not re-establish β₂-spectrin but still are capable of restoring InsP₃ receptor localization and normal contraction frequency of ankyrin-B⁻/⁻ cardiomyocytes. Therefore, direct binding of β₂-spectrin to ankyrin-B is required for the normal targeting of β₂-spectrin in neonatal cardiomyocytes. In contrast, ankyrin-B localization and function are independent of β₂-spectrin.

The finding that β₂-spectrin requires ankyrin-B for cellular targeting in cardiomyocytes was not anticipated. Studies of assembly of the erythrocyte skeleton have concluded that ankyrin associates with a preassembled spectrin skeleton (25). Moreover, β₂-spectrin has ankyrin-independent membrane binding sites (19, 27–29) and also associates with α-catenin (30). The current findings do not exclude the possibility that ankyrin-independent interactions of β₂-spectrin with proteins and phospholipids also contribute to its localization and function. It will be of
interest to evaluate localization of mutant forms of \( \beta_2 \)-spectrin lacking these activities.

A second surprising conclusion of this work is that \( \beta_2 \)-spectrin and ankyrin-B are in an intracellular compartment completely distinct from Golgi. Polypeptides immunoreactive with ankyrin and spectrin have been observed associated with Golgi at the level of immunofluorescence (31–33). However, the source of the spectrin-related Golgi staining recently has been

**Fig. 7.** Colocalization of intracellular ankyrin-B and \( \beta_2 \)-spectrin in neonatal cardiomyocytes. A–C, localization of ankyrin-B and \( \beta_2 \)-spectrin in neonatal cardiomyocytes derived from wild-type mice. The panels in A represent two-dimensional images of cardiomyocyte; the scale bar equals 10 \( \mu \)m. The panels in B and C are three-dimensional images constructed from consecutive Z-scans at 0.18 \( \mu \)m. The scale bar equals 2 \( \mu \)m. Note that both ankyrin-B and \( \beta_2 \)-spectrin are localized intracellularly.

**Fig. 8.** Ankyrin-B and \( \beta_2 \)-spectrin do not overlap with markers for the Z-line, T-tubule, or sarcoplasmic reticulum in three dimensions. Seven-day cardiomyocytes cultures were immunostained with antibodies to A, ankyrin-B (red) and \( \alpha \)-actinin (green); B, ankyrin-B (red) and dihydropyridine receptor (green); C, \( \beta_2 \)-spectrin (red) and InsP\(_2\) receptor (green); and D, \( \beta_2 \)-spectrin (green) and ryanodine receptor (red). Images are three-dimensional reconstructions of 10–20 Z-scans through the cardiomyocyte at 0.18-\( \mu \)m intervals.
identified as nesprin-1/nesy-1B (34), a large protein with spectrin repeats but not a member of the $\beta_2$-spectrin family (35). $\beta_2$-Spectrin has been associated with markers for the Golgi (36). In addition, 119-kDa ankyrin-G is localized in a subcellular pattern that overlaps with Golgi markers in subconfluent Madin-Darby canine kidney cells (37). It will be of interest to evaluate localization of $\beta_2$-spectrin and 119-kDa ankyrin-G using the three-dimensional rendering and high resolution confocal microscopy methods employed in this work.

A third unexpected finding was that $\beta_2$-spectrin is not required for ankyrin-B-dependent activity in targeting InsP$_3$ receptors in cardiomyocytes. Spectrin and ankyrin function collaboratively in stabilizing the plasma membrane of erythrocytes (12–14). Moreover, the phenotype of $\beta_{IV}$ spectrin-deficient mice is similar although less severe than ankyrin-G mutant mice (3, 4, 15). It is important to emphasize that our results were obtained using cultured neonatal cardiomyocytes and may not necessarily extend to neonatal heart tissue or to adult cardiomyocytes. It is of interest in this regard that loss of the sole $\beta_2$-spectrin ortholog in C. elegans is compatible with normal embryonic morphogenesis but not with survival after hatching (38, 39). Moreover, mice homozygous for a null mutation in $\beta_2$-spectrin display cardiac abnormalities as well as other defects and die in midgestation, consistent with a critical role for $\beta_2$-spectrin in the heart (40). One possible role for $\beta_2$-spectrin in cardiomyocytes would be to provide a mechanical protection for the ankyrin-B compartment during cardiac contractions. Such a stabilizing function would not necessarily be important under conditions of tissue culture but could be critical in the context of an actively beating heart.

This work defines the minimal spectrin binding domain as a sequence highly conserved in ankyrin polypeptides and encompassing a predicted ZU5 domain (smart.embl-heidelberg.de). This domain is also present in the tight junction protein ZO-1 and Unc5 netrin receptor (41, 42), although the level of homology is low, and these proteins do not have spectrin binding activity (data not shown). Interestingly, the computer-predicted ZU5 domain in ankyrin-B (residues 966–1070) did not interact with $\beta_2$-spectrin. However, full-spectrin binding occurred when we extended the boundaries of the ZU5 domain to 966–1125 based on a similar sequence flanking the ZU5 domain of Unc5. Ankyrins associate with $\beta_1$- and $\beta_2$-spectrins with 2–3-fold different affinities (19, 43), which raises the question of whether the minimal spectrin binding domain retains the ability to distinguish between spectrin isoforms. It is of interest in this regard that deletion of the N-terminal residues of the ankyrin-R spectrin binding domain results in a 20-fold loss of affinity from 10 to 200 nM (24), which would still register as a positive in yeast two-hybrid assays. It will be important to compare binding affinities of the minimal and full-length spectrin binding domains in biochemical assays.

In summary, this work demonstrates that interaction between members of the ankyrin and $\beta_2$-spectrin families previously established in erythrocytes and axon initial segments also occurs in neonatal cardiomyocytes with ankyrin-B and $\beta_2$-spectrin. This work also establishes a functional hierarchy in which ankyrin-B determines the localization of $\beta_2$-spectrin and operates independently of $\beta_2$-spectrin in its role in organizing membrane-spanning proteins. The supportive downstream role for $\beta_2$-spectrin in neonatal cardiomyocytes is consistent with results in C. elegans, where loss of spectrin is compatible with normal embryonic development. Such a “division of labor” between ankyrin and spectrin makes teleological sense considering the high level of diversity in protein interactions of ankyrin caused by ANK repeats and the properties of spectrin that allow formation of two-dimensional networks with actin. In contrast to erythrocytes and neurons, where ankyrins and spectrins are attached to the plasma membrane, the ankyrin-B-$\beta_2$-spectrin complex of cardiomyocytes is associated with intracellular membranes. The ankyrin-B-$\beta_2$-spectrin

FIG. 9. Ankyrin-B and $\beta_2$-spectrin staining is distinct from endoplasmic reticulum, Golgi, and early endosome markers in three dimensions. Seven-day cardiomyocytes cultures immunostained with antibodies to $\beta_2$-spectrin and Golgin 97 (left), ankyrin-B and GRP78/BiP (center), and ankyrin-B and EEA1 (right) are shown. The scale bar equals 5 $\mu$m. The bottom panel shows images of localization of early endosome marker EEA1 with ankyrin-B immunostaining. Images are three-dimensional reconstructions of 10–20 Z-scans through the cardiomyocyte at 0.18-$\mu$m intervals.
membrane compartments at the level of light microscopy. Eluci-
dation of the composition and functions of the ankyrin-B-β2-
spectrin compartment as well as its evolutionary connection
with the plasma membrane will be important goals for future
research.

Acknowledgments—We thank Jan Hoffman and Sarah Jones for
technical assistance.

REFERENCES
1. Mohler, P. J., Gramolini, A. O., and Bennett, V. (2002) J. Cell Sci. 115, 1565–1566
2. Bennett, V., and Baines, A. J. (2001) Physiol. Rev. 81, 1353–1392
3. Zhou, D., Lambert, S., Malen, P. L., Carpenter, S., Boland, L. M., and Bennett, V. (1998) J. Cell Biol. 143, 1295–1304
4. Jenkins, S. M., and Bennett, V. (2001) J. Cell Biol. 155, 739–746
5. Kizhatil, K., and Bennett, V. (2004) J. Biol. Chem. 279, 16706–16714
6. Mohler, P. J., Schott, J. J., Gramolini, A. O., Dilly, K. W., Guatimosim, S.,
   dufell, W. H., Song, L. S., Haurogne, K., Kyndt, F., Ali, M. E., Rogers, T. B.,
   Lederer, W. J., Escande, D., Le Marec, H., and Bennett, V. (2003) Nature 421, 634–639
7. Mohler, P. J., Saplwski, I., Napolitano, C., Bottelli, G., Sharpe, L., Timothy, K.,
   Priori, S. G., Keating, M. T., and Bennett, V. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9137–9142
8. Yu, J., and Goodman, S. R. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2340–2344
9. Luna, E. J., Kidd, G. H., and Brantont, D. (1979) J. Biol. Chem. 254, 2526–2532
10. Bennett, V. (1979) J. Biol. Chem. 253, 2292–2299
11. Bennett, V., and Stenbuck, P. J. (1979) J. Biol. Chem. 254, 2533–2541
12. Eber, S. W., Gonzalez, J. M., Lux, M. L., Scarpa, A. L., Tse, W. T., Dornwell, M.,
   Herbers, J., Kugler, W., Oncan, R., Pekrun, A., Gallagher, P. G., Schroter, W.,
   Forget, B. G., and Lux, S. E. (1996) Nat. Genet. 14, 214–218
13. Hanspal, M., Yoon, S. H., Yu, H., Hanspal, J. S., Lambert, S., Palek, J., and
   Prchal, J. T. (1991) Blood 77, 165–173
14. Bodine, D. M., 4th, Birkenmeier, C. S., and Barker, J. E. (1984) Cell 37, 721–729
15. Komada, M., and Soriano, P. (2002) J. Cell Biol. 156, 337–348
16. Mohler, P. J., Hoffman, J. A., Davis, J. Q., Abdi, K. M., Kim, C. R., Jones, S. K.,
   Davis, L. H., Roberts, K. F., and Bennett, V. (2004) J. Biol. Chem. 279, 25786–25804
17. Mohler, P. J., Davis, J. Q., Davis, L. H., Hoffman, J. A., Michaely, P., and
   Bennett, V. (2004) J. Biol. Chem. 279, 12980–12987
18. Mohler, P. J., Gramolini, A. O., and Bennett, V. (2002) J. Biol. Chem. 277, 10599–10607
19. Davis, J. Q., and Bennett, V. (1984) J. Biol. Chem. 259, 13550–13559
20. Hayes, N. V., Scott, C., Heerkens, E., Ohanian, V., Magno, A. M., Pinder, J. C.,
   Kordeli, R., and Baines, A. J. (2000) J. Cell Sci. 113, 2023–2034
21. Hu, R. J., Morothy, S., and Bennett, V. (1995) J. Cell Biol. 125, 1069–1080
22. Tovia, S., Bubusi, M., Davis, L., Reedy, M., and Bennett, V. (1999) J. Cell Biol. 147, 995–1008
23. Kennedy, S. P., Warren, S. L., Forget, B. G., and Morrow, J. S. (1991) J. Cell
   Biol. 115, 267–277
24. Davis, L. H., and Bennett, V. (1999) J. Biol. Chem. 265, 10589–10596
25. Woods, C. M., and Lazarides, E. (1988) Annu. Rev. Med. 39, 107–122
26. Wang, D. S., and Shaw, G. (1995) Biochem. Biophys. Res. Commun. 217, 608–615
27. Lombardo, C. R., Weed, S. A., Kennedy, S. P., Forget, B. G., and Morrow, J. S.
   (1994) J. Biol. Chem. 269, 29212–29219
28. Steiner, J. P., and Bennett, V. (1988) J. Biol. Chem. 263, 14417–14425
29. Steiner, J. P., Walke, H. T., and Bennett, V. (1989) J. Biol. Chem. 264, 2783–2791
30. Pradhan, D., Lombardo, C. R., Roe, S., Rimm, D. L., and Morrow, J. S. (2001)
   J. Biol. Chem. 276, 4175–4181
31. Beck, K. A., and Nelson, W. J. (1998) Biochim. Biophys. Acta 1404, 153–160
32. De Matteis, M. A., and Morrow, J. S. (1998) Curr. Opin. Cell Biol. 10, 542–549
33. De Matteis, M. A., and Morrow, J. S. (2000) J. Cell Sci. 113, 2331–2343
34. De Matteis, M. A., and Morrow, J. S. (2000) J. Biol. Chem. 275, 21419–21424
35. Apel, E. D., Lewis, R. M., Grady, R. M., and Sanes, J. R. (2000) J. Biol. Chem.
   275, 31986–31995
36. Stankewich, M. C., Tse, W. T., Peters, L. L., Ching, Y., John, K. M., Stabaich,
   P. R., Devarajan, P., Morrow, J. S., and Lux, S. E. (1998) Proc. Natl. Acad.
   Sci. U. S. A. 95, 14158–14163
37. Devarajan, P., Stabaich, P. R., Mann, A. S., Ardito, T., Kashgarian, M.,
   and Morrow, J. S. (1996) J. Cell Biol. 133, 819–830
38. Morothy, S., Chen, L., and Bennett, V. (2000) J. Cell Biol. 149, 915–930
39. Hammarlund, M., Davis, W. S., and Jorgensen, E. M. (2000) J. Cell Biol. 149,
   931–942
40. Tang, Y., Katuri, V., Deng, C. X., and Bennett, V. (2000) Proc. Natl. Acad.
   Sci. U. S. A. 97, 2769–2774
41. Ackerman, S. L., Kozak, L. P., Przyborski, S. A., Rund, L. A., Boyer, B. B., and
   Knowles, B. B. (1997) Nature 386, 838–842
42. Leonardo, E. D., Hinck, L., Masu, M., Keino-Masu, K., Ackerman, S. L., and
   Tessier-Lavigne, M. (1997) Nature 386, 838–848
43. Bennett, V., Davis, J., and Fowler, W. E. (1982) Nature 299, 126–131
Ankyrin-B Targets β2-Spectrin to an Intracellular Compartment in Neonatal Cardiomyocytes
Peter J. Mohler, Woohyun Yoon and Vann Bennett

*J. Biol. Chem. 2004, 279:40185-40193.*
doi: 10.1074/jbc.M406018200 originally published online July 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406018200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/07/23/M406018200.DC1

This article cites 43 references, 32 of which can be accessed free at
http://www.jbc.org/content/279/38/40185.full.html#ref-list-1