The Ankyrin-B C-terminal Domain Determines Activity of Ankyrin-B/G Chimeras in Rescue of Abnormal Inositol 1,4,5-Trisphosphate and Ryanodine Receptor Distribution in Ankyrin-B (−/−) Neonatal Cardiomyocytes*

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Ankyrins are a closely related family of membrane adaptor proteins that are believed to participate in targeting diverse membrane proteins to specialized domains in the plasma membrane and endoplasmic reticulum. This study addresses the question of how individual ankyrin isoforms achieve functional specificity when co-expressed in the same cell. Cardiomyocytes from ankyrin-B (−/−) mice display mis-localization of inositol 1,4,5-trisphosphate receptors and ryanodine receptors along with reduced contraction rates that can be rescued by expression of green fluorescent protein (GFP)-ankyrin-B but not GFP-ankyrin-G. We developed chimeric GFP expression constructs containing all combinations of the three major domains of ankyrin-B and ankyrin-G to determine which domain(s) of ankyrin-B are required for ankyrin-B-specific functions. The death/C-terminal domain of ankyrin-B determined activity of ankyrin-B/G chimeras in localization in a striated pattern in cardiomyocytes and in restoration of a normal striated distribution of both ryanodine and inositol 1,4,5-trisphosphate receptors as well as normal beat frequency of contracting cardiomyocytes. Further deletions within the death/C-terminal domain demonstrated that the C-terminal domain determined ankyrin-B activity, whereas deletion of the death domain had no effect. C-terminal domains are the most divergent between ankyrin isoforms and are candidates to encode the signal(s) that enable ankyrins to selectively target proteins to diverse cellular sites.

Ankyrins are a ubiquitously expressed family of membrane adaptor proteins that link a diverse set of membrane-associated proteins (including the anion exchanger, voltage-gated Na⁺ channel, Na⁺/K⁺ ATPase, and the L1 family of cell adhesion molecules) to the spectrin-based membrane skeleton (1). Vertebrate ankyrins are derived from three genes including ankyrin-R (ankyrin-1), ankyrin-B (ankyrin-2), and ankyrin-G (ankyrin-3) that encode multiple variants due to alternatively spliced transcripts of each gene product (1). Members of the ankyrin family have a high degree of sequence similarity, share common structural domains, and are often co-expressed in the same cells. For example, ankyrin-R, ankyrin-B, and ankyrin-G isoforms are co-expressed in brain, cardiac and skeletal muscle, and kidney (2, 3).

The results of ankyrin gene-specific knockouts in mice demonstrate that ankyrin isoforms have divergent and non-overlapping functions. For example, mutant mice with a cerebellar-specific knockout of ankyrin-G that still show normal expression of ankyrin-B and ankyrin-R in the cerebellum display a loss of voltage-gated Na⁺ channels and neurofascin from axon initial segments, a decreased ability of Purkinje neurons to fire action potentials, and a progressive ataxia (4). Similarly, mice deficient in ankyrin-B exhibit down-regulation and mis-sorting of calcium-release channels in cardiac and skeletal muscle and cardiac arrhythmias, despite the fact that ankyrin-R and ankyrin-G isoforms are normally expressed in both cardiac and skeletal muscle (2, 4–6). Furthermore, ankyrin-B null mice display significant central nervous system defects including reduced expression of L1 cell adhesion molecule, dilated ventricles, optic nerve degeneration, along with hypoplasia of the corpus callosum and pyramidal tracts (7), even though ankyrin-R and ankyrin-G are normally expressed in many of these areas of the brain (4, 8). Therefore, unlike a number of other protein families that can compensate for lack of specific isoform expression, ankyrin gene functions appear to be specific and are not compensated by other similar gene products. An important but currently unanswered question regarding these similar molecules is the basis for specificity in the unique functions of ankyrins.

Ankyrins are modular proteins composed of three major domains (see Fig. 4). The membrane-binding domains of ankyrins -B, -G, and -R are each composed of 24 consecutive ANK repeats. While possessing the same basic folding structure and 74% amino acid identity (human ankyrin-B compared with rat ankyrin-G), the membrane-binding domains of ankyrins exhibit functional differences in binding assays. For example, ankyrin-R and ankyrin-B display up to 10-fold differences in affinities for kidney microsomes (9). Likewise, ankyrin-B and ankyrin-R membrane-binding domains also have significant differences in affinities for the anion exchanger (10, 11). Therefore, the membrane-binding domain for each ankyrin has evolved for efficient function in specific cellular roles. Ankyrins also share a relatively conserved spectrin-binding domain with 68% amino acid identity between ankyrin-B and ankyrin-G. Spectrin-binding domains provide specificity for spectrin sub-
type association (i.e. ankyrin-R and β1-(erythroid) spectrin compared with ankyrin-B and 190-kDa spectrin (12, 13)). Finally, ankrys all share a domain containing a conserved death domain of unknown function (59% amino acid identity between ankyrin-B and ankyrin-G) and a C-terminal domain that is highly divergent across the ankyrin family (11% amino acid identity between ankyrin-B and ankyrin-G).

Transfection with ankyrin-B, but not ankyrin-G, can restore normal localization of calcium-release channels in the ER/SR of ankyrin-B (14). This study demonstrates, using ankyrin-B/G chimeras, that the death/C-terminal domain of ankyrin-B defines the subcellular localization of 220-kDa ankyrin-B as well as activity in restoring normal inositol 1,4,5-trisphosphate receptor (IP3R) and ryanodine receptor (RyR) localization and cardiomyocyte contractility. We further report that within the death/C-terminal domain, the C-terminal domain, and not the death domain, determines ankyrin-B activity. These findings indicate that ankyrin-B domains function in an integrated fashion and provide the first insights into how ankrys can selectively target proteins to diverse cellular sites.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Cardiomyocytes were dissociated from 1- to 2-day-old ankyrin-B (−/−) (7) or wild type littermates as described previously (14). For transfection assays, purified endotoxin-free DNA was isolated using Qiagen MidiPreps (Qiagen, Valencia, CA) and transfected using Effectene (Qiagen) or Genefector (Venn Nova, Pompano Beach, FL) according to manufacturer’s guidelines into 3–4-day-old myocyte cultures or cultured HEK293 cells. Concentrations and time for assays were determined empirically to ensure high population transfection efficiency (>70%) together with a moderate level of single cell expression (results not shown).

Plasmids—pEGFP (CLONTECH; Palo Alto, CA) 220-kDa ankyrin-B and 190-kDa ankyrin-G chimeric expression constructs were engineered using common molecular techniques. Briefly, an internal EcoRI site in ankyrin-B was removed by Quickchange PCR (Stratagene; La Jolla, CA). Next, pEGFPC2 and pEGFPN3 were modified to create a novel PmeI site in the pEGFP multiple cloning site. The membrane-binding domains of 220-kDa ankyrin-B and 190-kDa ankyrin-G were amplified by PCR to engineer a 5′ EcoRI site and 3′ AscI site resulting in a 3-amino acid linker (Gly-Ala-Pro) between the membrane- and spectrin-binding domains. The spectrin-binding domains of 220-kDa ankyrin-B and 190-kDa ankyrin-G (which lack the serine/threonine-rich insert and tail of 270-kDa ankyrin-G) were amplified by PCR to engineer a 5′ PacI site and 3′ AscI site resulting in a 3-amino acid linker (Leu-Ile-Asn) between the spectrin-binding and death/C-terminal domains. Finally, the death/C-terminal domains of ankyrin-B and ankyrin-G were amplified to contain 5′ PacI and 3′ PmeI sites. Amplified constructs were inserted into pNEB193 (New England Biolabs; Beverly, MA) and subsequently ligated into the modified pEGFP vectors using the available EcoRI and PmeI sites to create full-length GFP 220-kDa ankyrin-B and 190-kDa ankyrin-G expression constructs, as well as six other full-length ankyrin-B and ankyrin-G chimeras (Fig. 4). Similar methods
were utilized to create additional constructs (see Figs. 7A and S1A). All plasmids were verified first by restriction digestions and sequencing (ABI Prism; Duke DNA Core Facility), subsequently expressed in HEK293 cells (American Type Culture Collection (ATCC); Manassas, VA), and immunoblotted using GFP-specific antisera (CLONTECH) to ensure full-length protein products.

Quantitation of Spontaneous Contractions—Cardiomyocyte contraction rates (beats per min) were analyzed using differential interference contrast microscopy. Data represent at least three separate experiments (using at least three different mice), with a minimum of 50 cardiomyocytes analyzed per group. Myocytes were subsequently fixed in paraformaldehyde, and GFP-ankyrin chimera expression was confirmed by immunostaining (GFP-specific antisera) and confocal microscopy as described below.

Immunofluorescence and Immunoblotting—Primary cultures were fixed in 2% paraformaldehyde, permeabilized, and incubated with primary antibodies including green fluorescent protein (mouse, rabbit, and chicken; Chemicon, Temecula, CA), α-actinin (mouse; Sigma), IP3 receptor (type 1, rabbit; type 2, rabbit; Pan-antibody; rabbit), ryanodine receptor (type 2; mouse), SERCA2 (mouse; Affinity Bioreagents, Cambridge, UK), or ankyrin-B (mouse and rabbit), followed by appropriate secondary antisera (Alexa 488, 568; Molecular Probes; Sunnyvale, CA), and analyzed by confocal microscopy. Images at each wavelength (488 and 568 nm) were collected separately to ensure that there was no fluorescent channel bleed through. GFP ankyrin-transfected HEK293 cells were processed for SDS-PAGE and Western blotting as described (7) using GFP-specific antisera (Chemicon). All rescue experiment images are representative of hundreds of transfected myocytes in each culture. Experiments were repeated a minimum of three times using different neonatal mice.

Statistics—Data were analyzed using either paired two-tailed Student’s t tests or two-way ANOVA, and p values less than 0.05 were considered significant. Error bars in figures represent S.E.

RESULTS

Ankyrin-B Localization Precedes Calcium Homeostasis Protein Organization in Cultured Neonatal Cardiomyocytes—The goal of this study was to identify the ankyrin domain(s) that determine the difference between ankyrin-B and ankyrin-G in their cellular localization and the ability to restore localization of IP3R and RyR in primary cultures of ankyrin-B (−/−) cardiomyocytes from 1- to 2-day-old neonatal mice. The first step in establishing a rescue assay was to define culture conditions where ankyrin-B, IP3R, and RyR are normally organized. Cardiomyocytes were not examined before 24 h of culture, because at this stage cells were not yet firmly adhered to the coverslip and were routinely spherical (15). However, in 2-day-old cardiomyocyte cultures, cells were firmly attached, and α-actinin displays a costameric pattern as revealed by the normal Z-line localization pattern (Fig. 1). At this early developmental stage, ankyrin-B is striated and highly concentrated at the A-band.

In addition, some ankyrin-B staining (~15–20% of the total level of ankyrin-B) co-localizes with α-actinin, as well as in small (~0.5 μm) punctate structures throughout the cardiomyocyte (Figs. 1 and 2A). In day 2 cultures, the organization of the SR is poorly developed, as evidenced by the diffuse, punctate distribution of ryanodine receptor (RyR), IP3 receptor (IP3R), and the SR/ER calcium ATPase (SERCA2; Fig. 1, left-most portion of panel). Components of the calcium-release/uptake machinery of the SR begin to organize at ~4 days in culture (see SERCA2 staining); however, complete organization of ryanodine and IP3 receptor calcium-release channels does not display a more defined pattern until postnatal day 6 and 7. These developmental data also correlate with our observations that normal calcium waves and cellular contractions take several days to display a rhythmic, fluid characteristic (data not shown). The early expression and organized distribution of ankyrin-B in neonatal cardiomyocytes are consistent with a role of ankyrin-B in subsequent organization of IP3 and ryanodine receptors, which become localized only later in cardiomyocyte development.

These developmental time course experiments provided a framework for rescue studies using transiently transfected GFP expression constructs (see below) (Fig. 5). Because all of the cardiac molecules examined display a normal striated pat-
tern by 6–7 days in culture, we chose this developmental stage to address the effect of expression constructs on receptor channel localization. Therefore, cultures were transfected with the various GFP expression constructs at day 4–5 and subsequently analyzed at 6–7 days in culture.

GFP-Ankyrin Constructs Display Localization Patterns That Are Similar to Endogenous Ankyrins—The next step in establishing a system for evaluating ankyrin-dependent rescue of IP$_3$R and RyR localization in cardiomyocytes was to develop methods for transfection of cardiomyocytes resulting in normal localization of GFP-tagged ankyrin-B and ankyrin-G. We generated 220-kDa ankyrin-B and 190-kDa ankyrin-G cDNAs that were either N- or C-terminally fused with enhanced green fluorescent protein (pEGFP; Fig. 4, A and B). GFP expression was adjusted to low levels such that the GFP signal could only be detected by immunofluorescent staining with GFP antibody. Endogenous ankyrin-B in wild type cardiomyocytes localizes mainly at the A-band, with lower levels at the Z-line (Fig. 1 and Fig. 2A, upper panel). Similar to endogenous ankyrin-B, both the N- and C-terminal GFP fusions of 220-kDa ankyrin-B are primarily localized to the A-band in transfected ankyrin-B null cardiomyocytes with a less intense signal observed at the Z-line (Fig. 2A; shown are data using both the N-terminal GFP fusion, referred to as nGFP-ankyrin-B, and the C-terminal construct, cGFP-ankyrin-B). Endogenous ankyrin-G in wild type myocytes displays a diffuse membrane expression (Fig. 2B, upper panel) which is also seen using both N- and C-terminal GFP-fusions of 190-kDa ankyrin-G (Fig. 2B, lower panel).

These data establish that transfected GFP-ankyrin-B and ankyrin-G are expressed and targeted to identical localization patterns as their endogenous counterparts under our experimental conditions. In addition, these experiments demonstrate that GFP fused at either end of the proteins or the presence of the additional six linker residues (between the three major ankyrin domains; see under “Experimental Procedures”) do not interfere with the steady-state localization of either GFP 220-kDa ankyrin-B or 190-kDa ankyrin-G. Evidence that transfections under these conditions are not toxic to cardiomyocytes is that reduced contraction rates of ankyrin-B (−/−) cells is restored by transfection with ankyrin-B constructs (see below).

GFP 220-kDa Ankyrin-B Rescues IP$_3$ and Ryanodine Receptor Localization and Restores Normal Rates of Contraction in Ankyrin-B (−/−) Cardiomyocytes—Ryanodine and IP$_3$ receptors (Fig. 3, B and C), but not other calcium homeostasis proteins (including SERCA2, triadin, calreticulin, or the dihydropyridine receptor) are mis-localized in ankyrin-B (−/−) cardiomyocytes.$^2$ Transfection with plasmids encoding GFP-tagged 220-kDa ankyrin-B (both N- and C-terminal fusions; Fig. 3B), but not GFP alone (data not shown), restores the normally striated distribution of both ryanodine and IP$_3$ receptors in neonatal ankyrin-B null cardiomyocytes. In contrast, transfection with plasmids encoding GFP-tagged 190-kDa ankyrin-G does not restore a striated localization of either IP$_3$ or ryanodine receptor, which exhibit localization patterns in transfected cells resembling mock or untransfected phenotypes (Fig. 3C).

We measured spontaneous contraction rates of ankyrin-B (−/−) cardiomyocytes before and after transfection to assess the physiological impact of restoring IP$_3$R and RyR localization in ankyrin-B-deficient cardiomyocytes. Wild type cardiomyocytes contract rhythmically from a central perinuclear point at 144 ± 10 spontaneous beats per min (bpm; $n = 8$ mice, $>20$ cells averaged/mouse), whereas ankyrin-B (−/−) cardiomyocytes contract at 42 ± 3 bpm ($p < 0.05$, $n = 5$; Fig. 4). We next measured spontaneous contraction rates of ankyrin-B (−/−) cultures transfected with GFP-220-kDa ankyrin-B or GFP-190-kDa ankyrin-G. Analysis of GFP-ankyrin expression by immunofluorescence using GFP-specific antisera demonstrated that transfection efficiencies were 60–80% (Fig. 4). Therefore, a large number of cardiomyocytes were monitored to arrive at statistically significant sample populations. Ankyrin-B (−/−) cultures transfected with GFP, either C- or N-terminally fused to 220-kDa ankyrin-B contract at a rate similar to that of wild type cultures (129 ± 3 bpm; $p > 0.05$, $n = 4$ mice, $>80$ cells counted/mouse). In contrast to the normal beat frequency observed in ankyrin-B-transfected cells, ankyrin-B null myocytes

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$^2$P. J. Mohler, A. O. Gramolini, and V. Bennett, unpublished observations.
ANOVA. At least three times with greater than 50 myocytes measured per or untransfected control was measured. Experiments were performed spontaneous contractions were monitored. For each experiment, a mock, GFP, ankyrin-B or ankyrin-G (H11002 microscopy. Ankyrin-B (H11002) staining.

B

FIG. 4. 220-kDa GFP ankyrin-B restores normal contraction rates to ankyrin-B (−/−) cardiomyocytes. A, GFP immunofluorescence of GFP ankyrin-B-transfected cardiomyocytes highlighting transfection efficiency. Inset shows high power magnification of striated ankyrin-B staining. B, spontaneous contractions of wild type or ankyrin-B (AnkB) null (−/−) cardiomyocytes were monitored by light microscopy. Ankyrin-B (−/−) myocytes were transfected with ankyrin-B or ankyrin-G (AnkG) constructs, and 24–36 h later, spontaneous contractions were monitored. For each experiment, a mock, GFP, or untransfected control was measured. Experiments were performed at least three times with greater than 50 myocytes measured per culture. *, significant differences from wild type levels, p < 0.05, ANOVA.

expressing either C- or N-terminal GFP fusions of 190-kDa ankyrin-B display contraction rates that are not different from untransfected cardiomyocytes (32 ± 2 bpm, n = 4). As expected, the beat frequencies of mock-transfected (37 ± 4 bpm, n = 3) or GFP-transfected myocytes (41 ± 5 bpm, n = 3) are not significantly different from untransfected null cultures. These results are consistent with the lack of striated patterns of IP₃ and ryanodine receptor distribution in these cultures (Fig. 3). Taken together, these results demonstrate that the abnormal beat frequency characteristics displayed by ankyrin-B null cardiomyocytes are the direct result of a single molecular defect, because the reintroduction of 220-kDa ankyrin-B into these cultures restores normal contraction rates.

The Ankyrin-B C-terminal Domain Determines Activity of Ankyrin-B/G Chimeras in Subcellular Localization and Rescue of IP₃ and Ryanodine Receptor Distribution—We generated ankyrin-B/G chimeric constructs with the eight possible combinations of 220-kDa ankyrin-B and 190-kDa ankyrin-G membrane-binding, spectrin-binding, and death/C-terminal domains (Fig. 5B) to determine the essential domain(s) required for native ankyrin-B targeting and ability to rescue IP₃R and RyR localization in ankyrin (−/−) cardiomyocytes. Each construct (containing three amino acid linkers between domains) was N- and C-terminally fused with pEGFP resulting in a total of 16 ankyrin-B/ankyrin-G chimeras. Western blot analysis of cultured HEK293 cells transfected with these constructs using GFP-specific antisera revealed that ankyrin-B/G expression constructs generate proteins that migrate at the expected molecular weight (Fig. 5C).

GFP-tagged ankyrin-B/ankyrin-G chimeric constructs were transfected into 4–5-day-old ankyrin-B (−/−) cardiomyocytes and the localizations of the GFP-ankyrin chimera as well as the IP₃ and ryanodine receptor were subsequently examined in the same cells by double-label immunofluorescence. For these experiments, either N- or C-terminal fusions of GFP-ankyrin chimeras yielded identical findings; results for the remaining portion of the manuscript show data using the N-terminally fused constructs, i.e. those with GFP located at the C terminus of ankyrins.

Expression constructs where only the membrane-binding domains of ankyrin-B and ankyrin-G have been switched (i.e. comparing full-length ankyrin-B, M₈S₆D₃C₀, and M₈S₆D₃C₽) not only show very similar subcellular localization but both appear equally capable of restoring IP₃R and RyR localization in ankyrin-B (−/−) cultures (compare Fig. 3A and Fig. 6, top left panel). Conversely, the substitution of the ankyrin-B membrane-binding domain onto an ankyrin-G backbone (construct M₈S₆D₃C₀) results in a non-striated pattern of GFP expression and lack of activity in restoring IP₃R or RyR localization (Fig. 6). Together, these results demonstrate that the membrane-binding domains of 220-kDa ankyrin-B and 190-kDa ankyrin-G are interchangeable in this rescue assay, even though these domains share only 74% amino acid identity in their primary sequences.

Analysis of GFP constructs containing switched ankyrin-B and ankyrin-G spectrin-binding domains reveal that this domain is also interchangeable in our targeting/rescue assay. For example, chimeric ankyrin M₈S₆D₃C₀ is localized at the A-band and is also capable of restoring the localization of both ryanodine and IP₃ receptors (Fig. 6, left panel, middle). Similarly, the spectrin-binding domain of ankyrin-B within the ankyrin-G membrane binding domain and death/C-terminal domain (M₈S₆D₃C₀) is not capable of restoring the normal localization of ankyrin, IP₃, or ryanodine receptor (Fig. 6, right panel, top), further demonstrating that the spectrin-binding domain is not the unique region of the molecule that confers normal localization and calcium-channel rescue specificity to 220-kDa ankyrin-B.

The specificity of 220-kDa ankyrin-B targeting and activity in directing localization of IP₃R and RyR both reside within the death/C-terminal domain. The presence of the ankyrin-B death/C-terminal domain in the context of ankyrin-G (M₈S₆D₃C₀) can completely restore normal localization of GFP-ankyrin as well as of ryanodine and IP₃ receptors (Fig. 6, left panel, bottom). Furthermore, inclusion of the ankyrin-G death/C-terminal domain within the ankyrin-B construct (M₈S₆D₃C₀) abolished subcellular striated patterns for GFP and the ability to restore IP₃ or ryanodine receptor localization (Fig. 6, right panel, bottom). Together, these data clearly show that the death/C-terminal domain of 220-kDa ankyrin-B is required both for the localization of ankyrin and for normal IP₃R and RyR localization in cardiac muscle.

We next designed GFP fusion constructs containing only the death/C-terminal domain of either ankyrin-B or ankyrin-G (Fig. 7A, left), and we confirmed their activity in directing protein expression in HEK293 cells (Fig. 7A, right). Transfection of the death/C-terminal domain of 220-kDa ankyrin-B into
null cultures does not restore the normal distribution of IP₃ or ryanodine receptor (Fig. 7B), indicating that the death/C-terminal domain of 220-kDa ankyrin-B is necessary (see Fig. 5) but not sufficient for rescue of calcium-release channel localization. As expected, transfection of the ankyrin-G death/C-terminal domain construct into ankyrin-B (−/−) myocytes also

![Image](https://example.com/image1.png)

**FIG. 5.** Ankyrin-B/G chimeric GFP-tagged expression constructs. A, shown schematically are major protein domains of 220-kDa ankyrin-B (top) and 190-kDa ankyrin-G (bottom). Scores represent percent amino acid identity between ankyrin-G and ankyrin-B within the corresponding regions. B, chimeric constructs were generated within the pEGFP backbones (N-terminally fused, represented by n in the construct name; and C-terminally fused, represented by c) and contain various combinations of the membrane-binding, spectrin-binding, and death/C-terminal domains of 220-kDa ankyrin-B (gray) and 190-kDa ankyrin-G (black). C, GFP-ankyrin chimeric constructs were confirmed by sequencing and protein expression in HEK293 cells using GFP-specific antisera. Numbers represent the corresponding constructs in B. −, untransfected cells; lane GFP, cells transfected with empty GFP vector.

![Image](https://example.com/image2.png)

**FIG. 6.** Ankyrin-B/ankyrin-G chimeras containing the death/C-terminal domain of ankyrin-B restore IP₃R and RyR localization in ankyrin-B (−/−) cardiomyocytes. 4–6-Day-old ankyrin-B (−/−) cardiomyocyte cultures were transfected with GFP-ankyrin MₙSₙDCₙ, GFP-ankyrin MₘSₘDCₘ, GFP-ankyrin MₙSₙDCₙ, GFP-ankyrin MₘSₘDCₘ, or GFP-ankyrin MₙSₙDCₙ. Following 24–36 h, cultures were fixed and processed for immunofluorescence and confocal microscopy using antisera against α-actinin (results not shown), GFP, ryanodine, or IP₃ receptors. Data are representative of hundreds of transfected myocytes from four different mice using both N- and C-terminal GFP fusions. Bar equals 7.5 μm. M, membrane-binding domain; S, spectrin-binding domain; DC, death/C-terminal domain.
does not restore normal localization to either ryanodine or IP₃ receptors (Fig. 7C).

We next constructed ankyrin-B expression plasmids with deletions of either the death domain or the C-terminal domain (Fig. 8A). These constructs were sequenced, and expression of the correct molecular weight GFP fusion was confirmed in HEK293 cells (Fig. 8B). Ankyrin-B (-/-) cardiomyocytes were transfected with the ankyrin-G GFP-death/C-terminal domain construct (B) and the ankyrin-B death/C-terminal domain construct (C) and subsequently immunostained with antisera to GFP and ryanodine receptor or IP₃ receptor. Representative images were taken from three separate experiments, each using at least three mice. Note that although these constructs are expressed, they are unable to restore IP₃ or ryanodine receptor localization. Scale bar, 5 μm.

The Ankyrin-B C-terminal Domain Is Necessary for Activity of Ankyrin-B/G Chimeras in Restoration of Normal Contraction Rates of Ankyrin-B (-/-) Cardiomyocytes—Transfection of GFP-ankyrin M₄S₉DC₉ restores ankyrin-B (-/−) cardiomyocyte contraction rates nearly to levels observed in wild type cultures (Fig. 9; 124 ± 3 bpm; p = 0.05; n = 4 mice). By contrast, transfection of ankyrin M₄S₉DC₉ does not restore normal contractility to ankyrin-B (-/-) cardiomyocytes (41 ± 1 bpm, n = 4 mice; Fig. 9). These results are in agreement with immunolocalization studies demonstrating requirement of the death/C-terminal domain for activity of ankyrin-B/G chimeras in restoring localization of IP₃R and RyR (Fig. 5). We also determined that constructs encoding only the C-terminal domains of ankyrin-G and ankyrin-B fail to restore wild type contractility rates to null cultures (38 ± 4 and 36 ± 3 bpm, respectively, n = 3 mice; Fig. 9). Finally, transfection of cardiomyocytes with 220-kDa ankyrin-B lacking only the death domain largely restores normal beat frequency (126 ± 3 bpm; n = 4 mice) to ankyrin-B (-/−) myocytes. However, cardiomyocytes expressing GFP-ankyrin-B lacking the C-terminal domain...
domain of ankyrin-B (Fig. 9; 46 ± 1 bpm; n = 4 mice) did not display contractility properties that were significantly different from ankyrin-B (−/−) cardiomyocyte cultures (Fig. 9; 42 ± 2 bpm, n = 5). These results confirm the requirement of the ankyrin-B C-terminal domain, but not the death domain, in the rescue of IP₃ and ryanodine receptor localization and normal contraction rates ankyrin-B (−/−) cardiomyocytes.

**DISCUSSION**

This study reports that the C-terminal domain of 220-kDa ankyrin-B is necessary for activity of ankyrin-B/G chimeras for proper localization and for restoring the localization of IP₃ and ryanodine receptors as well as normal contraction rates to ankyrin-B null cardiomyocytes. The C-terminal domain is not active in the absence of membrane-binding and spectrin-binding domains, indicating that one or more of these domains must cooperate to ensure normal function of ankyrin-B. A major role for the C-terminal domain was initially surprising due to expected activities of the membrane-binding and spectrin-binding domains for ankyrin linkages to spectrin and membrane-associated proteins. However, as multiple ankyrin isoforms are commonly expressed within the same tissue, share closely related membrane- and spectrin-binding domains, but have non-overlapping functions, the C-terminal domains of other ankyrin isoforms may have similar regulatory functions that dictate isoform targeting and binding specificity within the ankyrin family.

Functions of the ankyrin-B C-terminal domain could result, in principle, from intramolecular and/or intermolecular interactions. Evidence for the potential of the C-terminal domain to participate in intramolecular interactions with other ankyrin domains comes from biochemical analysis of the C-terminal domain of ankyrin-R (16, 17). An ankyrin-R variant, lacking 161 residues in the C-terminal domain due to alternative splicing, has an increased affinity for spectrin and the anion exchanger. Moreover, the 161-residue segment binds directly to ankyrin-R, but not to individual spectrin- and membrane-binding domains, and can reverse the increased binding affinity for the anion exchanger. These observations led to the proposal that the 161 residues within the C-terminal domain of ankyrin-R bind to a site on ankyrin encompassing both membrane-binding and spectrin-binding domains and function as an allosteric repressor (17). Ankyrin-B contains a segment (residues 1600–1760) with limited sequence similarity to the 161 residues of ankyrin-R, although the possibility of alternatively spliced forms lacking this domain has not been evaluated.

**FIG. 9.** Ankyrin-B/G chimeras containing the ankyrin-B C-terminal domain restore normal cardiomyocyte contraction rates to ankyrin-B (−/−) cardiomyocytes. Neonatal myocytes were transfected with the indicated constructs (see Figs. 5, 7A, and 8A), and following 24–36 h, spontaneous contractions were monitored by light microscopy. In each experiment, a mock, GFP, or untransfected control was always included (see Fig. 4). Experiments were performed at least three times with greater than 50 myocytes monitored/culture. * significant differences from wild type levels, p < 0.05, ANOVA.

**FIG. 10.** Comparison of amino acid sequences of the death/C-terminal domains of ankyrin-B and ankyrin-G. The death/C-terminal domains of 220-kDa ankyrin-B (upper) and 190-kDa ankyrin-G (lower), which contain the death domain and the C-terminal domain, were aligned using MacVector (Accelrys; Burlington, MA). Shaded boxes represent conserved homology between these two molecules. The solid dark line above the sequence delineates the death domain. Arrows represent predicted cAMP-dependent protein kinase phosphorylation sites; open diamonds represent predicted protein kinase C phosphorylation sites; and the asterisk shows one predicted tyrosine phosphorylation site.
To date, no published information is available regarding intermolecular interactions of the death/C-terminal domain of 220-kDa ankyrin-B or other ankyrins. Moreover, searches of the available data bases with the ankyrin-B C-terminal domain sequence have not revealed homologies to known protein domains or binding sites. One feature of the C-terminal domain potentially relevant to protein interactions is a predicted amphipathic helix (1778–1788) that could mediate interactions with GTPases or protein kinases (18, 19).

Phosphorylation of the 220-kDa ankyrin-B C-terminal domain may also provide a mechanism for ankyrin-B-dependent localization of calcium-release proteins in the ER/SR membrane. It is of particular interest that multiple putative phosphorylation sites are predicted within this domain including 2 protein kinase A, 7 protein kinase C, 16 casein kinase II, and 1 tyrosine phosphorylation site (Fig. 10; Expasy Prosite, www.expasy.org).

The cellular mechanism involved in 220-kDa ankyrin-B-dependent localization of IP₃ and ryanodine receptor to the SR is currently unknown. The fact that 220-kDa ankyrin-B and IP₃ or ryanodine receptors do not demonstrate extensive co-localization argues against a simple stoichiometric 1:1 association between 220-kDa ankyrin-B and these receptors at the membrane of the sarcoplasmic reticulum. Immunoprecipitation studies have demonstrated interactions between IP₃R and ankyrins from native brain tissue and cultured cells (20–22). It will be important in future experiments to address the nature of ankyrin-B/calcium-release channel interactions to determine whether these are direct or indirect and if they are modulated by the C-terminal domain.

In conclusion, the results from this rescue study indicate that the C-terminal domain of ankyrin-B is critical for the normal localization of ankyrin-B, IP₃R, and RyR in neonatal cardiomyocytes. Extrapolation of these findings suggests that C-terminal domains in other ankyrins may also be required for dictating specificity for ankyrin function in diverse tissues. Both ankyrin-G and ankyrin-B isoforms have been implicated in the delivery of proteins to specialized membrane sites (4, 23, 24). However, unlike ankyrin-G polypeptides that have been characterized at plasma membranes in various cell types, ankyrin-B appears to be intimately involved in the regulation ER/SR calcium compartment. Therefore, these isoforms appear to have similar but non-overlapping roles in the organization of protein complexes. Based on the results of the current study, we speculate that the divergent roles of ankyrins are dependent on their C-terminal domains.

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