Ankyrin-G and $\beta_2$-Spectrin Collaborate in Biogenesis of Lateral Membrane of Human Bronchial Epithelial Cells*

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Ankyrins are a family of adapter proteins required for localization of membrane proteins to diverse specialized membrane domains including axon initial segments, specialized sites at the transverse tubule/sarcoplasmic reticulum in cardiomyocytes, and lateral membrane domains of epithelial cells. Little is currently known regarding the molecular basis for specific roles of different ankyrin isoforms. In this study, we systematically generated alanine mutants of clusters of charged residues in the spectrin-binding domains of both ankyrin-B and -G. The corresponding mutants were evaluated for activity in either restoration of abnormal localization of the inositol trisphosphate receptor in the sarcoplasmic reticulum in mutant mouse cardiomyocytes deficient in ankyrin-B or in prevention of loss of lateral membrane in human bronchial epithelial cells depleted of ankyrin-G by small interfering RNA. Interestingly, ankyrin-B and -G share two homologous sites that result in loss of function in both systems, suggesting that common molecular interactions underlie diverse roles of these isoforms. Ankyrins G and B also exhibit differences; mutations affecting spectrin binding had no effect on ankyrin-B function but did abolish activity of ankyrin-G in restoring lateral membrane biogenesis. Depletion of $\beta_2$-spectrin by small interfering RNA phenocopied depletion of ankyrin-G and resulted in a failure to form new lateral membrane in interphase and mitotic cells. These results demonstrate that ankyrin-G and $\beta_2$-spectrin are functional partners in biogenesis of the lateral membrane of epithelial cells.

Physiological functions of most membrane-spanning proteins of metazoa require that the proteins localize within specialized domains within the plasma membrane or endoplasmic reticulum. Emerging evidence from animal models and human mutations suggests that one of the mechanisms for organizing functionally related subsets of proteins in specialized membrane domains operates through the ankyrin family of membrane adapters (1). Ankyrin-G is required for neurons to be able to fire action potentials and for the clustering of Na$_v$1.6, KCNQ2/3 channels, and the L1 cell adhesion molecule neurofascin at axon initial segments (2–5). A human mutation in the ankyrin-G-binding site of Na$_v$1.5 blocks its clustering at intercalated discs and transverse tubule sites in cardiomyocytes and causes a cardiac arrhythmia (6). Moreover, ankyrin-G is required for targeting the RhBG ammonium transporter to basolateral domains of kidney epithelial cells (7).

Ankyrin-B is required for localization of InsP$_3$ receptors at specialized sites within the sarcoplasmic reticulum of cardiomyocytes (8–11). Ankyrin-B-dependent clusters of InsP$_3$ receptors in the sarcoplasmic reticulum are coincident with Na/K-ATPase and Na$^+$/Ca$^{2+}$ exchanger on the transverse tubules and are a specialized feature of cardiomyocytes not found in skeletal muscle (12). Ankyrin-B mutations that block the ability to segregate InsP$_3$ receptors Na/K-ATPase and Na$^+$/Ca$^{2+}$ exchanger cause a dominantly inherited fatal cardiac arrhythmia (10, 12, 13).

Mechanisms for ankyrin-based targeting of membrane proteins have been explored in cultured epithelial cells (14–16). Early studies led to a proposal of an outside-in hierarchy of protein interactions where homotypic adhesion of E-cadherin at sites of cell-cell contact results in recruitment of spectrin followed by ankyrin and its partner, the Na/K-ATPase (16). Recent siRNA-based methods for inhibiting protein expression have allowed direct tests of the role of ankyrin in membrane protein organization. A surprising result of elimination of 190-kDa ankyrin-G was that luminal epithelial cells no longer formed a lateral membrane and developed a squamous morphology (17). These results suggest that ankyrin-G directly participates in de novo biogenesis of the lateral membrane.

Ankyrin-B and ankyrin-G are overall quite similar in amino acid sequence, with the major differences primarily residing in their C-terminal regulatory domains (9, 18, 19). The high level of sequence similarity in membrane-binding and spectrin-binding domains of ankyrin-B and -G suggests the possibility of conserved mechanisms underlying their apparently distinct functions. In this study, we have further compared the spectrin-binding domains of ankyrin-B and ankyrin-G by performing systematic alanine-scanning mutagenesis to determine functionally important amino acid residues predicted to be accessible to solvent. Such surface-ex-
posed residues would be available for protein-protein interactions. We identified two homologous sites that are not required for spectrin binding or cellular targeting, but are essential for function of both ankyrins-B and -G. We have previously reported that ankyrin-B does not require β2-spectrin for localization of InsP3 receptors, although ankyrin-B is required for cellular targeting of β2-spectrin in cardiomyocytes (20). We demonstrate here that in contrast to ankyrin-B, ankyrin-G requires β2-spectrin for its function in epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Ankyrin Mutagenesis**—Clusters of charged residues predicted to be surface exposed were mutated to alanines in the spectrin-binding domain of human 220-kDa ankyrin-B and rat 190-kDa ankyrin-G. The A1024P mutant, which is comparable with the spectrin binding-defective A1000P mutation in ankyrin-B (20), was introduced into the ankyrin-G spectrin-binding domain. All mutations were introduced using the QuikChange XL site-directed mutagenesis kit (Strategene). DNA sequencing confirmed all the alanine mutations were present with no additional mutation in the spectrin-binding domain. The plasmids coding GFP-fused human ankyrin-B and GFP-fused rat ankyrin-G have been described (9).

Ankyrin-B mutants include: 1) ED865AA; 2) KE868AA; 3) DD872AA; 4) E892AA; 5) RSD895ASA; 6) DR905AA; 7) RD917AA; 8) DD923AA; 9) KE938AA; 10) ER941AA; 11) DAR953AA; 12) RH969AA; 13) RK996AA; 14) KR1010AA; 15) HR1012AA; 16) EGE1022AGA; 17) RGK1055AGA; 18) RER1065AA; 19) E1067AA; 20) D1070AA; 21) KE1073AA; 22) D1081AA; 23) DE1092AA; 24) ED1099AA; 25) EK1102AA; 26) KR1104AA; 27) RD1122AA; 28) KR1157AA; 29) KK1174AA; 30) K1180AA; 31) EPR1190APA; 32) RRK1193AAA; 33) KR1104AA; 34) ED1241AA; 35) RE1286AA; 36) K1302AA; 37) RD1338AA; 38) E1344AA; 39) HH1365AA; 40) D1385AA; 41) E1326AA; 42) E1329A; 43) R1336A; 44) RD1388AA; 45) EGK1344AGA; 46) HK1374AA; 47) KE1374AA; 48) RD1385AA; 49) KE1399AA; 50) K1402A; 51) E1427A; 52) E1431A; and 53) EEE1433AAA.

Ankyrin-G mutants include: 1) ED865AA; 2) KE868AA; 3) EE878AA; 4) ED892AA; 5) KE938AA; 6) RD917AA; 7) KE985AA; 8) DD923AA; 9) KE938AA; 10) ER941AA; 11) DAR953AA; 12) RH969AA; 13) RK996AA; 14) KR1010AA; 15) HR1012AA; 16) EGE1022AGA; 17) RGK1055AGA; 18) RER1065AA; 19) E1067AA; 20) D1070AA; 21) KE1073AA; 22) D1081AA; 23) DE1092AA; 24) ED1099AA; 25) EK1102AA; 26) KR1104AA; 27) RD1122AA; 28) KR1157AA; 29) KK1174AA; 30) K1180AA; 31) EPR1190APA; 32) RRK1193AAA; 33) KR1104AA; 34) ED1241AA; 35) RE1286AA; 36) K1302AA; 37) RD1338AA; 38) E1344AA; 39) HH1365AA; 40) D1385AA; 41) E1326AA; 42) E1329A; 43) R1336A; 44) RD1388AA; 45) EGK1344AGA; 46) HK1374AA; 47) KE1374AA; 48) RD1385AA; 49) KE1399AA; 50) K1402A; 51) R1405A; 52) E1427A; 53) E1431AA; and 54) EEE1433AAA.

Cells and Transfection—Neonatal cardiomyocytes were isolated from the hearts of P1 ankyrin-B+/- mice and transfected as described previously (11, 20). Human bronchial epithelial cells were cultured and transfected as described (17). Briefly, the cells were trypsinized and replated twice in the 24-h period preceding seeding cells for transfection, a procedure empirically determined to enhance transfection efficiency. Then, 10^5 cells were seeded on the 1.4-mm coverslip of a MatTek tissue culture dish (MatTek). Twelve h after plating, cells were preincubated in a serum-free medium (Opti-MEM from Invitrogen) for 30 min to remove serum proteins that bind to Lipofectamine and to reduce the integrity of cell junctions. The cells, still in a serum-free medium, were then co-transfected with 1 μg of ankyrin-G siRNA plasmid or β2-spectrin siRNA plasmid and 0.1 μg of rescue plasmid using Lipofectamine 2000 (3.1 μg, Invitrogen). The 10-fold excess of siRNA over rescue plasmids ensures that cells expressing the rescue plasmid also will be transfected with siRNA plasmid. Hence, GFP (ankyrin-G rescue) or HA (β2-spectrin rescue) staining serves as a tracer for cells that have been transfected with the siRNA. This transfection strategy also results in a population of cells transfected with siRNA alone. These cells can serve as an internal control for siRNA-induced phenotypes in rescue assays. 16 h after transfection (26 h after plating), cells were analyzed by immunofluorescence. Lateral membrane height was visualized by determining the distance of the tight junction marked by ZO-1 from the base of the cell in an XZ plane.

**Immunoblot Analysis**—HEK 293 cells were transfected with ankyrin-B and ankyrin-G mutants using Lipofectamine 2000 (Invitrogen). Cell lysates were subjected to SDS-PAGE and immunoblot analysis with affinity-purified GFP antibody. Immunoblot analysis was used to evaluate β2-spectrin deple- tion. 60-mm dishes were seeded with cells at 4 x 10^5 cells and transfected with 4 μg of β2-spectrin siRNA plasmid after 12 h. pSuper and missense siRNA plasmids were transfected into cells as controls. Cell lysates were immunoblotted with β2-spectrin antibody 18 h after transfection. Primary antibodies were detected using 125I-labeled protein A/G as described (9, 17).
Immunofluorescence—Cells were fixed and immunostained with antibodies as described previously (10, 17). Mouse neonatal cardiomyocytes were fixed with 4% paraformaldehyde and blocked and permeabilized with 0.075% Triton X-100 and 3% fish oil gelatin (Sigma) in phosphate-buffered saline. Human bronchial epithelial cells were fixed with 2% paraformaldehyde and incubated with 10% horse serum and 0.1% Triton X-100 in phosphate-buffered saline. Then, the cells were incubated with primary antibodies overnight at 4°C and with fluorescence-conjugated secondary antibodies (Alexa 488 and 568, Molecular Probes) for 8 h at 4°C. The primary rabbit antibodies against ankyrin-B, InsP3 receptor, ankyrin-G, β2-spectrin, and GFP were described earlier (17, 20). Monoclonal antibodies against ZO-1 (Zymed Laboratories Inc.) and Na/K-ATPase (Affinity Bioreagents) were commercially obtained. The gp135 hybridoma supernatant was a gift of Dr. G. Ojakian (SUNY Down State Medical Center, Brooklyn, NY). Images were collected with a laser scanning confocal microscope using either a 63× NA 1.4 or a 100× NA 1.45 objective.

Yeast Two-hybrid Analysis—The yeast two-hybrid assay for association of the spectrin-binding domain of ankyrin-B with β2-spectrin has been described (20). The spectrin-binding domain (residues 874–1476) of rat 190-kDa ankyrin-G was PCR-amplified and inserted between the AscI and PacI sites of pAS2-1 (Clontech). The spectrin-binding domain alanine mutants from the ankyrin-B and the ankyrin-G mutants were subcloned into pAS2-1 plasmid (Clontech) using the AscI and PacI sites. Spectrin-binding domain mutants were co-transformed with the β2-spectrin vector into yeast (AH109) and analyzed for a positive interaction using dropout media, Ade/His/Leu/Trp and Leu/Trp, transformation control.

RESULTS

Alanine-scanning Mutagenesis of Charged Residues in the Spectrin-binding Domains of Ankyrin-B and Ankyrin-G—To map key residues on the surface of the spectrin-binding domain of ankyrin-B and the ankyrin-G mutants were cloned into pAS2-1 plasmid (Clontech) using the Ascl and PacI sites. Spectrin-binding domain mutants were co-transformed with the β2-spectrin vector into yeast (AH109) and analyzed for a positive interaction using dropout media, −Ade/His/Leu/Trp and −Leu/Trp, transformation control.

Immunofluorescence—Cells were fixed and immunostained with antibodies as described previously (10, 17). Mouse neonatal cardiomyocytes were fixed with 4% paraformaldehyde and blocked and permeabilized with 0.075% Triton X-100 and 3% fish oil gelatin (Sigma) in phosphate-buffered saline. Human bronchial epithelial cells were fixed with 2% paraformaldehyde and incubated with 10% horse serum and 0.1% Triton X-100 in phosphate-buffered saline. Then, the cells were incubated with charged clusters of two or more residues were replaced with alanines. The validity of using clustered charged residues to predict surface residues is exemplified in a study of actin where 81% of predicted surface residues were in fact located on the surface, based on the subsequent atomic structure (23). Fifty-four mutants were generated in ankyrin-B, and 47 mutants were generated in ankyrin-G (see Fig. 1A). DNA sequencing confirmed all the alanine mutations without any additional
mutations. Alanine mutation in principle may cause a structural change resulting in protein misfolding and loss of function. To exclude such structural mutations, the alanine mutants were expressed in HEK 293 cells and analyzed by immunoblot with GFP antibody. As shown in Fig. 2, all mutants of ankyrin-B and -G that resulted in loss of function (see below) generated full-length polypeptides when expressed in HEK 293 cells. In addition, all ankyrin-G mutants were targeted normally to the lateral membrane where endogenous ankyrin-G is localized in human bronchial epithelial cells (17) (Fig. 2C). With the exception of EGK1344AGA, all ankyrin-B mutants were appropriately expressed and properly targeted in ankyrin-B-/- neonatal cardiomyocytes (Figs. 2B and 3, GFP). These results indicate that the mutant ankryins are folded appropriately without major structural alterations.

**Loss-of-function Mutants of Ankyrin-B**—The alanine mutants of ankyrin-B were evaluated to determine whether each mutant was able to rescue abnormal InsP₃ receptor localization in mouse ankyrin-B-/- neonatal cardiomyocytes. Of the 54 mutants evaluated, we identified seven that were unable to rescue the InsP₃ receptor localization in ankyrin-B-/- cardiomyocytes (Fig. 3, D–J). These mutants include ED1099AA, EK1102AA, KR1104AA, DK1322AA, RD1338AA, EGK1344AGA, and E1427A. Previously, EDE1081AAA was characterized as a loss-of-function mutant in this cardiomyocyte system (20). All other ankyrin-B mutants (see RH985AA (Fig. 3C)) restored normal InsP₃ receptor phenotypes of ankyrin-B+/− cardiomyocyte. Interestingly, the eight loss-of-function mutations are clustered in three regions: EDE1081AAA/ED1099AA, EK1102AA, and KR1104AA are within the minimal spectrin-binding domain (Ser-966 to Lys-1125) (20); DK1322AA, RD1338AA, and EGK1344AGA comprise another cluster; and E1425A and E1427A are close to the site of the human mutation E1425G, which causes type 4 long QT syndrome (10).

**Loss-of-function Mutants of Ankyrin-G**—Rat ankyrin-G mutants were assessed in human bronchial epithelial cells where knockdown of 190-kDa ankyrin-G by RNA interference causes a loss of lateral membrane and an expansion of basal/apical membrane, changing columnar cells to a squamous morphology (Fig. 4, GFP). Co-expression of rat ankyrin-G, which has three different nucleotides within the human siRNA-targeted sequence and is resistant to human siRNA, prevents loss of lateral membrane (Fig. 4A, W7) (17). Unlike wild-type ankyrin-G-GFP and 43 other mutants, four
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mutants of ankyrin-G, DAR999AAA, EE1123AA, KR1128AA, and KD1363AA failed to preserve the lateral membrane following the depletion of ankyrin-G (Fig. 4B, white arrows). Localization of ZO-1, a tight junction protein, was included to identify the apical portion of the lateral membrane. GFP-immunostaining (green) was used to trace cells transfected with both the ankyrin-G siRNA and the rescue plasmids.

Interestingly, the ankyrin-G mutant EGK1369AGA was active in targeting and rescue assays (not shown), even though the corresponding ankyrin-B mutant EGK1344AGA was mislocalized in cardiomyocytes. This suggests that the ankyrin-B EGK1344AGA mutation does not cause misfolding and instead specifically affects targeting.

Ankyrin-B and Ankyrin-G Share Functional Residues Unrelated to Spectrin Binding—Interestingly, three critical sites of the spectrin-binding domain are shared between ankyrin-B and -G (Fig. 1). These common sites are ED1099/EE1123, KR1104/KR1128, and RD1338/KD1363 of ankyrin-B/G (Fig. 1). These sites are likely required for a function(s) that is not directly related to spectrin binding, as mutations did not affect spectrin interaction in yeast two-hybrid assays (see below, Fig. 5). In addition, these sites are well conserved not only in human ankyrin isoforms but also in ankyrins of Drosophila and Caenorhabditis elegans (Fig. 1B). In contrast to common sites, other loss-of-function mutants were isosom-specific sites, EDE1081, DK1322, EGK1344, and E1427 of ankyrin-B are specific to InsP₃ receptor targeting function in cardiomyocytes, whereas DAR999 of ankyrin-G is specific to lateral membrane biogenesis in epithelial cells, even though most of these residues are conserved between ankyrin-B and -G.

Loss of Spectrin Binding Abolishes Activity of Ankyrin-G—We next analyzed spectrin binding activity of loss-of-function mutants. The spectrin-binding domains from the loss-of-function mutants of ankyrin-B and ankyrin-G were subcloned into a pAS2-1 vector and tested for β₂-spectrin interaction in yeast two-hybrid assays. Positive interaction of the spectrin-binding domain with β₂-spectrin is displayed by growth of yeast on selection media (-Ade/His/Leu/Trp). We previously demonstrated that ankyrin-B mutants lacking spectrin binding activity also do not restore localization of β₂-spectrin in ankyrin-B-deficient cardiomyocytes, confirming the in vivo relevance of the yeast two-hybrid assay (20). The ankyrin-B/β₂-spectrin association, however, is not required for ankyrin-B-mediated InsP₃ receptor targeting, because the mutants lacking spectrin-targeting activity still restored normal InsP₃ receptor localization. Consistent with this finding, all the loss-of-function mutants of ankyrin-B retained spectrin binding activity in the yeast two-hybrid assay (Fig. 5A).

One ankyrin-G loss-of-function mutant, DAR999AAA, lost spectrin binding activity (Fig. 5B), whereas other loss-of-function mutants, EE1123AA, KR1128AA, and KD1363AA, retained the spectrin interaction. Ankyrin-B mutant DAR975AAA, corresponding to mutation of ankyrin-G at DAR999AAA, did not display loss of function in cardiomyocytes (20) but did display loss of spectrin binding activity (Fig. 5). We previously reported that A1000P mutation of ankyrin-B, identified in a search for mutations affecting spectrin binding, abolished spectrin binding activity (20). A1000P mutation of ankyrin-B abolished the ability to direct β₂-spectrin targeting in cardiomyocytes but had no effect on the ability of ankyrin-B to direct the targeting of InsP₃ receptors (20). We therefore evaluated the effects of the corresponding A1024P mutation in ankyrin-G on spectrin binding (Fig. 5B) and activity in restoring lateral membrane (Fig. 6). As expected, the A1024P mutant ankyrin-G lost the ability to bind spectrin in the yeast two-hybrid assay (Fig. 5B). However, in contrast to ankyrin-B, the A1024P mutation of...
ankyrin-G also abolished function in restoring lateral membrane in ankyrin-G-depleted epithelial cells. The A1024P mutant ankyrin-G retained activity in localization to the lateral membrane and was expressed as a full-length polypeptide (Fig. 6). Together, these results suggest that ankyrin-G, unlike ankyrin-B, is inactivated by mutations at two distinct sites that each result in loss of spectrin binding activity.

**β₂-Spectrin Is Required for Lateral Membrane Formation in Interphase and Mitotic Cells**—To determine whether β₂-spectrin, like ankyrin-G, is required for formation of the lateral membrane, we used siRNA to deplete the protein in HBE cells as determined by immunoblot analysis of cell extracts and by immunofluorescence (Fig. 7). Cell viability 16 h after transfection was essentially unaffected (96% survival based on trypan blue exclusion) but declined before.

Loss of β₂-spectrin results in reduction of the height of the lateral membrane from 7 to 1 μm, accompanied by a 3-fold increase in the cross-sectional area of apical and basal membranes (in Fig. 7, B and D, compare the horizontal distances between arrowheads in left and right panels). Cells were transfected after 12 h in culture when the height of the lateral membrane was 3–4 μm (not shown). The reduced height thus results from a combination of loss of pre-existing lateral membrane and the inability to accumulate additional lateral membrane. Apical-basal polarity is unaffected by depletion of β₂-spectrin based on the following findings. gp135 (Fig. 7D, compare top panels) retains its apical localization (24). The Na/K-ATPase was lost from the lateral membrane but did not mislocalize to the apical membrane (Fig. 7D, bottom panels). ZO-1 still was present at the border of the apical and lateral surfaces (Fig. 7B). Moreover, E-cadherin was still restricted to the lateral surface (not shown). HA-tagged β₂-spectrin, mutated at three sites in the siRNA targeting sequence, was resistant to siRNA knockdown and prevented loss of the lateral membrane (Fig. 7E, red).

Loss of β₂-spectrin thus phenocopies the consequences of depleting 190-kDa ankyrin-G, with loss of lateral membrane, expansion of apical and basal membrane areas, and preservation of apical-basal polarity. This result is consistent with earlier observations that expression of domains of β₂-spectrin disrupts morphology in cultured epithelial cells (25) and that β-spectrin is required for accumulation of Na/K-ATPase in Drosophila epithelial cells (26). The lack of requirement for β-spectrin in epithelial morphogenesis of C. elegans (27, 28) may reflect differences between nematodes and vertebrates or the fact that most epithelial cells in this organism do not have extensive lateral membranes.

New lateral membrane is rapidly synthesized in epithelial cells between anaphase and telophase. To evaluate the role of β₂-spectrin in this process of membrane biogenesis, we determined the effects of protein knockdown on the formation of lateral membrane during mitosis. Cells in late telophase, identified by the tubulin-rich midbody structure (Fig. 8A, red XY confocal section; white XZ confocal section), showed a well
The cells were labeled with 2-spectrin mutated at three wobble position sites to give a missense siRNA. B, 2-spectrin depletion in HBE cells results in loss of the lateral membrane accompanied by expansion of the apical and basal membranes, Top, XY confocal image; bottom, XZ confocal images; green, 2-spectrin, red, ZO-1. C, quantification of the reduction in lateral membrane and an increase in cross-sectional area of the cell following 2-spectrin depletion. The following plasmids were transfected into cells: pSuper (column 1); missense human 2-spectrin siRNA plasmid (column 2); human 2-spectrin siRNA plasmid (column 3). D, depletion of 2-spectrin does not affect apical polarity. XZ confocal images, sp135 (green, top) is retained at the apical membrane, and Na/K-ATPase (green, NKA, bottom) does not mislocalize to the apical membrane. E, exogenous 2-spectrin mutated at three sites in the siRNA targeting sequence (see “Experimental Procedures”) rescues the phenotype resulting from 2-spectrin depletion; XZ confocal images, 2-spectrin HA (red) and ZO-1 (green, arrowheads). Scale bars, 20 μm.

FIGURE 8. 2-spectrin knockdown prevents lateral membrane biogenesis in epithelial cells undergoing cytokinesis. A, XY and XZ confocal images of cells in the telophase stage, identified by the presence of the midbody stained with β-tubulin (white), that have been transfected with missense siRNA (left) and 2-spectrin siRNA (right). The cells were labeled with 2-spectrin (red) and β-catenin as a marker for the lateral membrane (green, arrows, see “Results”). Cells depleted of 2-spectrin fail to develop a lateral membrane between the daughter cells. B, defective lateral membrane biogenesis following 2-spectrin depletion in HBE cells results in a progressive accumulation of binucleate cells because of a failure to complete cytokinesis. Missense siRNA-transfected cells (black bars) and 2-spectrin siRNA-transfected cells (gray bars). Results are means ± S.D. Scale bars, 10 μm.

DISCUSSION

This study provides a systematic comparison of functional requirements of predicted surface residues of the spectrin-binding domain for ankyrin-B-mediated targeting of InsP_3 receptor to specialized endoplasmic reticulum/sarcoplasmic reticulum membrane in cardiomyocytes and ankyrin-G-mediated biogenesis of lateral membrane in epithelial cells. Loss-of-function mutants in the spectrin-binding domain of ankyrin-B and -G included two key sites commonly required for the function of both ankyrins and isoform-specific sites. The two conserved functionally important sites are not required for association with spectrin or for cellular localization of either ankyrin-B or ankyrin-G. However, these residues are highly conserved among all three human ankyrins as well as the ankyrins of *C. elegans* (Unc-44) and *Drosophila* (Fig. 1). Alanine mutations at these sites
do not cause a generalized loss of structure because mutant ankyrins are targeted normally in both cardiomyocytes and epithelial cells, are expressed as full-length polypeptides, and retain spectrin binding activity in yeast two-hybrid assays. These sites could participate in either intra- or inter-molecular interactions of the spectrin-binding domain. These sites may also be targets for phosphorylation. For example, the serine adjacent to the RD/KD site is conserved in all ankyrins (Fig. 1). It will be important in future work to identify protein(s) and possibly protein kinases that interact with these conserved sites.

This study provides several lines of evidence that ankyrin-G and β₂-spectrin collaborate in biogenesis of the lateral membrane of epithelial cells. DAR999AAA and A1024P mutations that abolish spectrin binding in yeast two-hybrid assays also abolish function in restoration of the lateral membrane in ankyrin-G-depleted epithelial cells. Moreover, knockdown of β₂-spectrin phenocopies the depletion of ankyrin-G in interphase cells, with loss of the lateral membrane, expansion of apical and basal membrane area, and conversion of cells from columnar to squamous morphology. In addition, knockdown of β₂-spectrin also prevents the formation of new lateral membrane during mitosis as previously reported for ankyrin-G-depleted cells (17).

The process of lateral membrane biogenesis in dividing cells has interesting parallels with cellularization in Drosophila embryos where new lateral membrane is rapidly formed in the space of minutes (29). Interestingly, lava lamp, a protein required for cellularization associates with spectrin as well as dynein and dynactin (30). It will be important to directly evaluate the roles of β-spectrin and ankyrin in the assembly of new lateral membrane in Drosophila embryos and, conversely, the roles of homologues of lava lamp in the formation of lateral membranes of mammalian epithelial cells.

β₂-Spectrin interacts with phosphatidylinositol lipids through its pleckstrin homology domain (31). Moreover, β-spectrins associate with membrane phospholipids through multiple sites (32, 33). Spectrins also interact with microtubule-based motors either directly (34) or through dynactin (33, 35). These properties of interaction with lipids and motor proteins have led to proposals that β-spectrin has a role in connecting membrane compartments with microtubule-based motors. A missing element in this model is a mechanism for specific association with membrane proteins. Ankyrins recognize multiple membrane proteins through ankyrin repeats and are capable of forming homo- and heterocomplexes between membrane proteins (12, 36). Ankyrin-G and perhaps other members of the ankyrin family thus are candidates to mediate sorting of membrane proteins for subsequent transport by a spectrin-microtubule pathway.

β₂-Spectrin is tightly associated with α₂-spectrin and also interacts with actin, protein 4.1, and adducin. β-Spectrin has been reported to function independently of α-spectrin in targeting the Na/K-ATPase to basolateral membranes of Drosophila epithelial cells (26). It will be of interest to determine the roles of α-spectrin and other β-spectrin-associated proteins in membrane biogenesis and, in particular, to determine whether they are involved in membrane trafficking and/or scaffolding roles. A working hypothesis is that proteins promoting an extensive spectrin-actin network would only operate at the lateral membrane and that β-spectrin would need to be maintained in an unassembled state prior to delivery to the lateral membrane.

Ankyrins and spectrins are colocalized in a variety of specialized cell sites including basolateral membranes of transporting epithelia (14, 37), nodes of Ranvier and initial segments of neurons (3, 38), as well as costameres (39) and neuromuscular junctions (40, 41) in striated muscle. Ankyrin-G and β₂-spectrin have been demonstrated to interact at axon initial segments (3, 38), although the precise ankyrin-spectrin partners remain to be identified in other cell types. Thus β-spectrins and ankyrins may function together in a conserved mechanism for the formation of diverse membrane domains.

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