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Ankyrin-Based Trafficking and Scaffolding of Membrane Proteins: Implications for Plasma Membrane Stability, Formation, and Specialization

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Abstract

Through the collaborative actions of β-spectrin and ankyrin, a cytoskeletal adaptor protein, integral and peripheral membrane proteins find order and stability in the relatively fluid environment of the plasma membrane. Not only is the ankyrin/β-spectrin complex responsible for the proper targeting and retention of membrane proteins but it facilitates the formation of multi-protein complexes to maximize local signaling between membrane and effector proteins. Dysfunction in ankyrin or β-spectrin causes deficiencies in fundamental cellular properties such as membrane stability, excitability, and adhesion. This review focuses on the direct effects of ankyrin function on membrane proteins in terms of binding and stability, intracellular transport, membrane targeting and retention, and altered biophysical properties. We propose that ankyrin and β-spectrin are important for the normal progression of many membrane proteins along their biosynthetic pathway from stabilizing the membrane protein to it's proper trafficking and eventual targeting and retention at membrane domains. The second half of the review addresses how an ankyrin/membrane protein interaction influences the local membrane environment with particular emphasis on membrane stability, membrane domain formation, and membrane domain specialization. We propose that not only are ankyrins necessary for erythroid membrane stability but they are required in some cells types for membrane domain formation and integral for the formation of specialized membrane domains in myocytes.

Keywords: Ankyrin; Spectrin; Intracellular trafficking; Protein targeting; Membrane scaffolding; Ion channels; Cardiac arrhythmia

Introduction

Membrane proteins facilitate a variety of interactions between the external and internal cellular environments including the transport or exchange of ions and molecules, cellular adherence to a surrounding substrate or neighboring cell, and the translation of an extracellular signal into an altered cellular response. Given the importance of membrane proteins, many cellular processes are involved in the delivery, retention, and recycling of these proteins. Ankyrins are adaptor proteins that link membrane proteins to the underlying cytoskeleton. Both ankyrin and its cytoskeletal cohort β-spectrin have been linked to many steps in the biosynthetic pathway of membrane proteins from intracellular transport to membrane targeting and retention, in addition to clathrin-mediated endocytosis and endosomal recycling. This review is organized around two central themes: ankyrin function on membrane proteins and the cellular effects of ankyrin/membrane protein interactions. The first aim will address the direct effect of ankyrin function on membrane proteins in terms of protein binding, intracellular trafficking, membrane targeting and retention, and altered biophysical properties. In the later half, a discussion on the cellular effects of ankyrin/membrane protein interactions will include the mechanical stabilization of plasma membrane, membrane domain formation, and membrane-domain specialization.

Ankyrins

Ankyrins serve as an interface between membrane-bound proteins and the underlying cytoskeleton. This interaction contributes to the stability of the membrane protein's location and expression within the plasma membrane. Ankyrins appear to be a metazoan invention as they have only been detected in worms, flies, rodents, and humans, but not in yeast or plants. In human, three genes *ANK1*, *ANK2*, and *ANK3* encode isoforms of ankyrin-R, ankyrin-B, and ankyrin-G respectively. While very little is known about the regulation of ankyrin gene transcription, it will be complex because ankyrin genes are quite large with multiple first exons and numerous alternative transcripts have already been identified. Alternative splicing of ankyrin genes results in a diverse array of isoforms with unique functions and distinct expression patterns. Expression of ankyrin-R isoforms has been detected in erythrocytes, striated muscle, and some neurons. In contrast, isoforms of ankyrin-B and ankyrin-G have been detected in a greater variety of tissues. While some tissues such as the heart and cerebellum display all three ankyrin gene products, they are not functionally redundant, i.e. ankyrin-G cannot compensate for the loss of ankyrin-B in cardiomyocytes [1].

The prototypical ankyrin has three functional domains: a membrane-binding domain (MBD), spectrin-binding domain (SBD), and a C-terminal regulatory domain (CTD) (Figure 1). The membrane-binding domain is comprised of 24 consecutive ANK repeats that are arranged in a superhelical array forming a solenoid [2]. The ANK repeats have inherent spring-link qualities that confer resilience to the membrane-binding domain from mechanical perturbations that occur

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in ankyrin-expressing tissues such as erythrocytes and striated muscle [3]. ANK repeats are a common motif for protein-protein interactions and most of ankyrin interactions with integral membrane proteins occur via the ANK repeats in the membrane-binding domain. The spectrin-binding domain contains a ZU5 domain (from the mouse zona occludens 1 (ZO-1) and the *C. elegans* uncoordinated protein 5 (unc5)) that comprises the minimal binding domain for spectrin [4]. In contrast, spectrin repeats 14 and 15 comprise the minimal binding domain for ankyrin [5-7]. In addition to spectrin, the spectrin-binding domain has been shown to interact with B56α, a regulatory subunit of protein phosphatase 2A, and dynactin-4, an adaptor protein that links the dynein motor to membrane cargo [8,9]. As different ankyrin gene products are relatively homologous within the MBD and SBD, the C-terminal regulatory domain, which is the least homologous, governs the specificity of ankyrin function and its subcellular localization. The CTD confers ankyrin specificity by regulating ankyrin interactions with itself, the cytoskeleton, and integral membrane proteins [10-12]. Not surprisingly, many of the missense mutations associated with ankyrin dysfunction have been localized to the C-terminal regulatory domain [13]. The prevalence of ankyrin dysfunction in a variety of human disorders including hemolytic anemia, cardiac arrhythmias, and neonatal diabetes highlights the significance of ankyrin function for normal cellular physiology [14-18].

Ankyrin functions

Binding and stabilization of membrane proteins: Ankyrin interacts with a variety of integral membrane proteins including ion channels, transporters, and cell adhesion molecules (see Table 1). Ankyrin-associated ion channels include voltage-gated sodium channels ($Na_V1.x$) and potassium channels (K_V3, K_V7), the L-type voltage-gated calcium channel $Ca_V1.3$, inositol triphosphate receptors (IP_3R), and the potassium inward rectifying channel subunit Kir6.2 [17,19-26]. Ankyrin-associated ion transporters include the sodium/calcium exchanger (NCX), sodium/potassium ATPase (NKA), anion exchangers (AE1, AE2, AE3), hydrogen/potassium ATPase, and the RhBG ammonium transporter [27-34]. Ankyrin-associated cell adhesion molecules include the family of L1-CAMs, E-cadherin, CD44, and β-dystroglycan [35-40]. While the structural requirements underlying many of these interactions have yet to be elucidated, previous studies have demonstrated that MBD ANK repeats mediate ankyrin interactions with integral membrane proteins. Some interactions involve one ANK repeat, while other interactions require multiple consecutive ANK repeats. For example, ankyrin-G ANK repeat 14 or 15 is sufficient to bind $Na_V1.5$, while the NCX binding site is spread across ankyrin-B ANK repeats 16, 17, and 18 [41,42]. One ankyrin molecule can interact with multiple membrane proteins simultaneously, thereby allowing for multi-protein complex formation. Another important aspect of ankyrin binding is that this interaction stabilizes the membrane protein. In ankyrin haploinsufficiency, ankyrin-associated membrane proteins including NCX, NKA, IP_3R, $Na_V1.5$, and $Ca_V1.3$ display reduced protein expression and membrane localization [21,42-44]. More detailed studies have demonstrated that ankyrin-B binding to the IP_3R lengthens the receptor's half-life from 3.7 hours to 11.7 hours [45]. Likewise, NCX binding to ankyrin-B extends the exchanger's half-life from 17.8 hours to 27.2 hours [41]. In summary, multiple membrane proteins can simultaneously bind to an ankyrin membrane-binding domain and this interaction stabilizes the membrane proteins.

Protein	Ankyrin	Domain
Ion channels:		
Rh antigen	R	
IP_3R	B	MBD
$Ca_V1.3$	B	
Kir6.2	B	MBD
$Na_V1.X$	G	MBD
K_V7	G	
K_V3	G	
CNG-β	G	
Ion transporters:		
Anion exchanger (AE1, 2, 3)	R	MBD
NCX	R, B	MBD
NKA	R, B, G	MBD
Ammonium transporter	R, G	
Cell adhesion molecules:		
CD44	R	MBD
L1-CAM family	R, B, G	MBD
E-cadherin	G	
β-dystroglycan	G	
Cytoskeletal/structure:		
β-spectrin	R, B, G	SBD
Obscurin	R, B, G	CTD
Dystrophin	B, G	
Filamin C	G	CTD
Plakophilin-2	G	
Plectin	G	CTD
Intracellular transport:		
Clathrin	R	MBD
Tubulin	R, B	MBD
EHD1-4	B	MBD
Dynactin-4	B	SBD
EB1/3	G	
Other:		
PP2A	B	SBD
Hdj1/Hsp40	B	CTD
Fas	G	DD
Tiam-1	R, G	MBD
Sigma receptor	B	

Table 1: Proteins that interact with ankyrins (R, B, or G) are grouped according to their general function. Sites of interaction on ankyrin are listed (MBD: membrane-binding domain, SBD: spectrin-binding domain, DD: death domain, CTD: C-terminal domain).

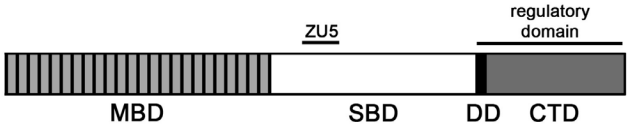

Figure 1: *Ankyrin functional domains*. The prototypical ankyrin has three functional domains. The membrane-binding domain (MBD) is comprised of 24 ANK repeats that bind many membrane proteins. The spectrin-binding domain (SBD) includes the ZU5 motif, which is the minimal binding domain for spectrin. The death domain (DD) and C-terminal domain (CTD) regulate ankyrin's association with itself, membrane proteins, and the cytoskeleton.

Intracellular trafficking of membrane proteins: While a lot of ankyrin biology research has focused on the targeting and retention of integral membrane proteins at the plasma membrane, there is evidence that ankyrin and β-spectrin interact with membrane proteins at many steps along the membrane protein's biosynthetic pathway. First, isoforms of ankyrin (Ank195 and AnkG119) and βspectrin (β3-

spectrin) have been associated with the trans Golgi network [46-52]. Furthermore, the association of β3-spectrin with the Golgi is partially regulated by an ADP ribosylation factor-dependent increase in the level of phosphatidylinositol 4,5-bisphosphate in the Golgi membrane [53]. β3-spectrin is connected to the molecular motor dynein through its interaction with actin-related protein (Arp1) that is a component of the dynactin complex [54]. Considering its interactions with the dynein/dynactin complex and its notable similarities to other coat-like proteins such as clathrin, β3-spectrin is thought to be involved in vesicular trafficking. Using a dominant negative construct to disrupt Golgi-targeting of the endogenous β-spectrin, it was demonstrated that the endoplasmic reticulum (ER) to Golgi transport of both the α- and β-subunits of NKA is dependent upon β3-spectrin [49]. In support of these findings, β3-spectrin null mice display a large number of vesicles around the Golgi [55]. Similar to β3-spectrin, ankyrin is involved in ER to Golgi transport of the NKA α-subunit. Ankyrin interacts with a specific binding domain in the α-subunit that is necessary for the subunit's ER to Golgi transport [56]. Furthermore, normal ER to Golgi trafficking of the α-subunit is disrupted by over-expression of this domain. In contrast, ER to Golgi trafficking is rescued by inclusion of this ankyrin-binding domain in a fusion protein that would have otherwise remained in the ER [56]. Other converging evidence in support of ankyrin's role in intracellular trafficking includes a direct interaction of ankyrin with tubulin [57-59] and dynactin-4 [8,35]. In addition, a new study demonstrated a direct interaction between ankyrin-G and the microtubule plus-end binding proteins EB1 and EB3 [60]. This interaction partially regulates ankyrin-G's subcellular localization at the axon initial segments of neurons, suggesting that ankyrin-G may play an important role at the dynamic interface of the plus-end microtubule and the actin cytoskeleton.

While both ankyrin and β-spectrin have been associated with microtubule-based transport, ankyrin is also connected to clathrin-mediated endocytosis and endosomal-based trafficking. Ankyrin-R MBD binds to clathrin [61]. In addition, ankyrin-B MBD interacts with all four of the Eps15-homology domain containing (EHD) proteins, which are involved in endosomal-based anterograde and retrograde trafficking of membrane proteins [62]. Even though all four EHD proteins are expressed in the heart, there appears to be a preferential interaction between ankyrin-B and EHD3 given that ankyrin-B haploinsufficiency caused the most significant up-regulation of EHD3 protein expression. In support of the hypothesis that EHD3 is involved in anterograde transport of ankyrin-B associated proteins, NCX membrane expression was increased by EHD3 over-expression and conversely its expression was decreased by EHD3 down-regulation [62]. Finally, a truncated isoform of ankyrin-G has been detected in late endosomal compartments that are immunopositive for the lysosomal-associated membrane glycoprotein, suggesting that there is a lysosomal-specific ankyrin isoform in addition to a Golgi-specific isoform [63].

Targeting and scaffolding of membrane proteins: One of the more obvious deficiencies associated with ankyrin dysfunction is the loss of membrane protein targeting and scaffolding. For example, ankyrin-B haploinsufficiency causes a decrease in the membrane expression of the L-type voltage-gated calcium channel $Ca_V1.3$ in both sinoatrial cells and atrial myocytes [16,21]. Interestingly, ankyrin-B is required for full membrane expression of $Ca_V1.3$ and decreased channel expression is associated with sinus node disease and atrial fibrillation [16,21]. In the nervous system, the intrinsic self-assembly of axon initial segments is predominantly mediated by ankyrin-G-dependent retention of voltage-gate sodium and potassium channels, as well as the cell adhesion molecule neurofascin-186.

The axon initial segment (AIS) is a highly specialized region of the neuron that initiates the action potential thereby facilitating electrical and chemical communication between neurons. Action potential initiation is achieved through the coordinated activities of a variety of voltage-gated ion channels clustered at the AIS. Ankyrin-G is critical for AIS-enrichment of voltage-gated sodium channels $Na_V1.2$ and $Na_V1.6$, in addition to the enrichment of potassium channel subunits KCNQ2 and KCNQ3 [20,22-24,64]. In the absence of ankyrin-G in cerebellar Purkinje neurons, voltage-gated sodium channels are no longer properly localized to AIS and the neurons display reduced action potential generation [64]. Ankyrin-G interaction with voltage-gated sodium channels is positively regulated by channel phosphorylation by the AIS-enriched CK2 kinase [65,66]. Ankyrin-G also targets cell adhesion molecules including neurofascin-186 and neuron glia-related cell adhesion molecule (NrCAM) to the AIS. In Purkinje neurons, neurofascin-186 is the target of synapse formation from GABAergic basket interneurons [67]. In hippocampal neurons, neurofascin-186 recruits the chondrotin sulfate proteoglycan brevican to the AIS, which has an inhibitory effect on interactions between pre- and post-synaptic membranes [68,69]. Ankyrin-G retention of voltage-gated ion channels and cell adhesion molecules is absolutely essential for AIS intracellular and extracellular formation.

Modulation of membrane protein biophysical properties: While the effect of ankyrin-binding on the biophysical properties of membrane proteins has not been studied in great detail, ankyrin has been shown to alter intrinsic biophysical properties of voltage-gated sodium channels and the potassium inward rectifying channel subunit Kir6.2. Mohler et al. [15] described a missense mutation in the ankyrin-binding domain of $Na_V1.5$ that disrupted channel association with ankyrin-G and was linked to Brugada syndrome, a cardiac disorder caused by decreased sodium current density. The missense mutation causes changes in activation and inactivation states of $Na_V1.5$ in a heterologous expression system. Similarly, Shirahata et al. [70] has demonstrated that ankyrin-G accelerates the rate of $Na_V1.6$ inactivation in a heterologous expression system. On the contrary, Lowe et al. [42] found no change in the inactivation state of $Na_V1.5$ following ankyrin-G knockdown in cardiomyocytes. These conflicting results warrant additional studies to clarify the effect of ankyrin-G on intrinsic properties of the voltage-gated sodium channel.

The potassium inward rectifying channel subunit (Kir6.2) is another membrane protein that has altered biophysical properties upon ankyrin-binding. Kir6.2 is an ATP-sensitive channel that links cellular metabolism with cellular excitability. Increased metabolism elevates intracellular ATP that binds to Kir6.2 and closes the channel, leading to membrane depolarization and cellular excitability. It has been shown that ankyrin-B selectively binds to the pore-forming channel subunit Kir6.2, but not to Kir6.1 [17,71]. Moreover, the ankyrin-B/Kir6.2 protein complex includes the regulatory sulfonylurea receptor subunits SUR1 and SUR2, although ankyrin-B does not directly bind to these regulatory subunits [17,71]. Ankyrin-B/Kir6.2 interaction enhances channel membrane expression and decreases the channel's ATP sensitivity [17,71]. The molecular mechanisms underlying the ankyrin-dependent decrease in ATP sensitivity have yet to be discovered, but the ankyrin-B interaction may cause steric hindrance between the ATP molecule and Kir6.2.

Cellular effects of ankyrin/membrane protein interactions

Maintenance of plasma membrane mechanical stability: The bicarbonate/chloride exchanger band 3 (or anion exchanger 1, AE1) is the most abundant membrane protein in erythrocytes. In addition to playing a key role in carbon dioxide transport in blood, the anion exchanger also serves as a point of attachment for the erythroid cytoskeleton. Membrane-bound AE1 predominantly exists as a dimer and tetramer. The dimer is attached to the cytoskeleton through the junctional complex with its principal components AE1, protein 4.1, p55, and glycophorin. In contrast, the tetramer is linked to the cytoskeleton through the ankyrin complex that contains the core subunits AE1, ankyrin-R, and protein 4.2. Mutations to proteins in the ankyrin complex are generally associated with hereditary sphereocytosis, a type of hemolytic anemia that is quite common, but renders the erythrocytes vulnerable to mechanical and osmotic disruption [72]. Of the subunits in the ankyrin complex, ankyrin-R mutations are the most predominant cause of hereditary sphereocytosis (HS). At the molecular level, disrupting any component of the ankyrin complex compromises the attachment between the erythroid membrane and its underlying cytoskeleton; therefore, ankyrin-R interactions with AE1 are critical for the normal conformation and stability of erythroid membranes.

Membrane domain formation: In some cell types, the interaction of ankyrin with cell adhesion molecules and cytoskeletal proteins is important for the formation and/or maintenance of membrane domains. Specifically, it has been demonstrated that ankyrin-G is necessary for lateral membrane biogenesis in bronchial epithelial cells [36,73]. Ankyrin-G directly interacts with the cytoplasmic domain of the cell adhesion molecule E-cadherin leading to the retention of E-cadherin and β-catenin at nascent adherens junctions in growing lateral membrane domains [73,74]. Ankyrin-G recruitment of β2-spectrin stabilizes the developing adherens junctions and allows for the accumulation of lateral membrane. When ankyrin-G or β2-spectrin is reduced by siRNA treatment, there is a complete loss of lateral membrane biogenesis and a compensatory increase in the apical and basal membranes [73,74]. Interestingly, the apical to basal polarity in these epithelial cells is maintained despite the loss of lateral membrane [73,74]. Not only does ankyrin-G and β2-spectrin stabilize the lateral membrane domain expression of E-cadherin, but they also play important roles in the transport of E-cadherin from the trans-Golgi network. Disruption of ankyrin-G/E-cadherin interactions significantly increases the mislocalization of E-cadherin to the trans-Golgi network [36]. Lateral membrane biogenesis in bronchial epithelial cells is dependent on the post-Golgi transport and membrane stabilization of E-cadherin by an ankyrin-G/β2-spectrin protein complex.

In the mammalian retina, ankyrin-G is also important for the biogenesis of outer segments of rod photoreceptors. Specifically, ankyrin-G-treated retinas displayed significantly shortened rod outer segments compared to the control-treated retinas [75]. While the molecular mechanisms underlying this ankyrin-G-dependent membrane biogenesis has yet to be characterized, future studies should focus on ankyrin-associated cell adhesion molecules.

Membrane domain specialization

Transverse-tubules: In cardiac ventricular myocytes, transverse-tubules (T-tubules) are invaginations of the plasma membrane that maximize the interface between the sarcolemma and extracellular milieu. They facilitate the rapid and efficient propagation of membrane depolarization to the myocyte interior thereby ensuring the rapid and synchronized release of intracellular calcium from the sarcoplasmic reticulum (SR). T-tubules are enriched with ion channels and transporters that mediate the transmembrane flux of calcium ions. Calcium-induced calcium release from the SR is predominantly regulated by the coordinated activities of the L-type voltage-gated calcium channel (or dihydropyridine receptor, DHPR) and the ryanodine receptor (RyR) (Figure 2). As an integral membrane protein in the T-tubule, DHPR is aligned opposite the RyR, a SR integral membrane protein, through actions of the pore-forming channel subunit [76] or the β1 auxiliary subunit [77,78]. The T-tubule is also enriched with NCX that acts in conjunction with the sarcoplasmic reticulum calcium ATPase (SERCA) to reduce cytosolic calcium levels during the myocyte relaxation phase. NCX is functionally coupled to NKA and the proper targeting/retention of this protein complex at the T-tubules is dependent upon interactions with ankyrin-B (Figure 2). Ankyrin-B directly binds NCX and NKA [43], an interaction that stabilizes NCX protein [41]. Ankyrin-B haploinsufficiency results in reduced NCX and NKA protein expression and membrane localization at T-tubules [41,43,44]. Reduced T-tubular NCX function increases post-systolic calcium levels in the cytosol, thereby enhancing SERCA's contribution to cytosolic calcium removal and resulting in elevated SR calcium stores [44]. Therefore, ankyrin-B-dependent targeting and retention of NCX and NKA at T-tubules contributes to the functional specialization of this domain, i.e. the normal efflux of calcium ions during the myocyte relaxation phase.

Intercalated disc: In ventricular cardiomyocytes, intercalated discs (ICD) are specialized domains that mediate the end-end contact between adjoining myocytes and allow for electrical and mechanical continuity between these cells. In the intercalated discs, desmosomes and adherens junctions function in the mechanical adhesion between neighboring myocytes, while connexons (or gap junctions) facilitate the electrical coupling between these cells. Each ICD junctional complex has distinct protein components with specialized functions; nevertheless, these complexes are interconnected and functionally dependent on each other. Ankyrin-G interacts with the desmosomal protein plakophilin-2 and the gap junction protein connexin43 (Figure 2). Decreasing ankyrin-G expression results in reduced ICD localization of plakophilin-2 and diminished intercellular adhesion [79]. Furthermore, reduced ankyrin-G expression causes a decrease in protein expression and ICD localization of connexin43 resulting in decreased junctional conductance [79]. Interestingly, ankyrin-G and plakophilin-2 appear to mutually facilitate their retention at the ICD because siRNA-mediated plakophilin-2 knockdown decreases the ICD localization of both ankyrin-G and the voltage-gated sodium channel $Na_V1.5$ [79]. Ankyrin-G scaffolding of plakophilin-2 and connexin43 contributes to the electromechanical coupling between adjoining cardiomyocytes.

The voltage-gated sodium channel $Na_V1.5$ initiates the rapid upstroke of the cardiac action potential. This channel displays differential subcellular localization in ventricular cardiomyocytes. While a small population of $Na_V1.5$ has been localized to lateral membranes, the most abundant population is localized at the ICD (Figure 2). $Na_V1.5$ differential localization arises from the channel's association with different protein complexes. Lateral membrane localization is the result of channel association with the syntrophin-dystrophin complex, while ICD localization of the channel is regulated by ICD-resident proteins synapse associated protein 97 (SAP97) and ankyrin-G. Syntrophin interacts with the PDZ domain encoded by the last three amino acids in $Na_V1.5$ (Ser-Ile-Val). Disrupting this interaction leads to decreased

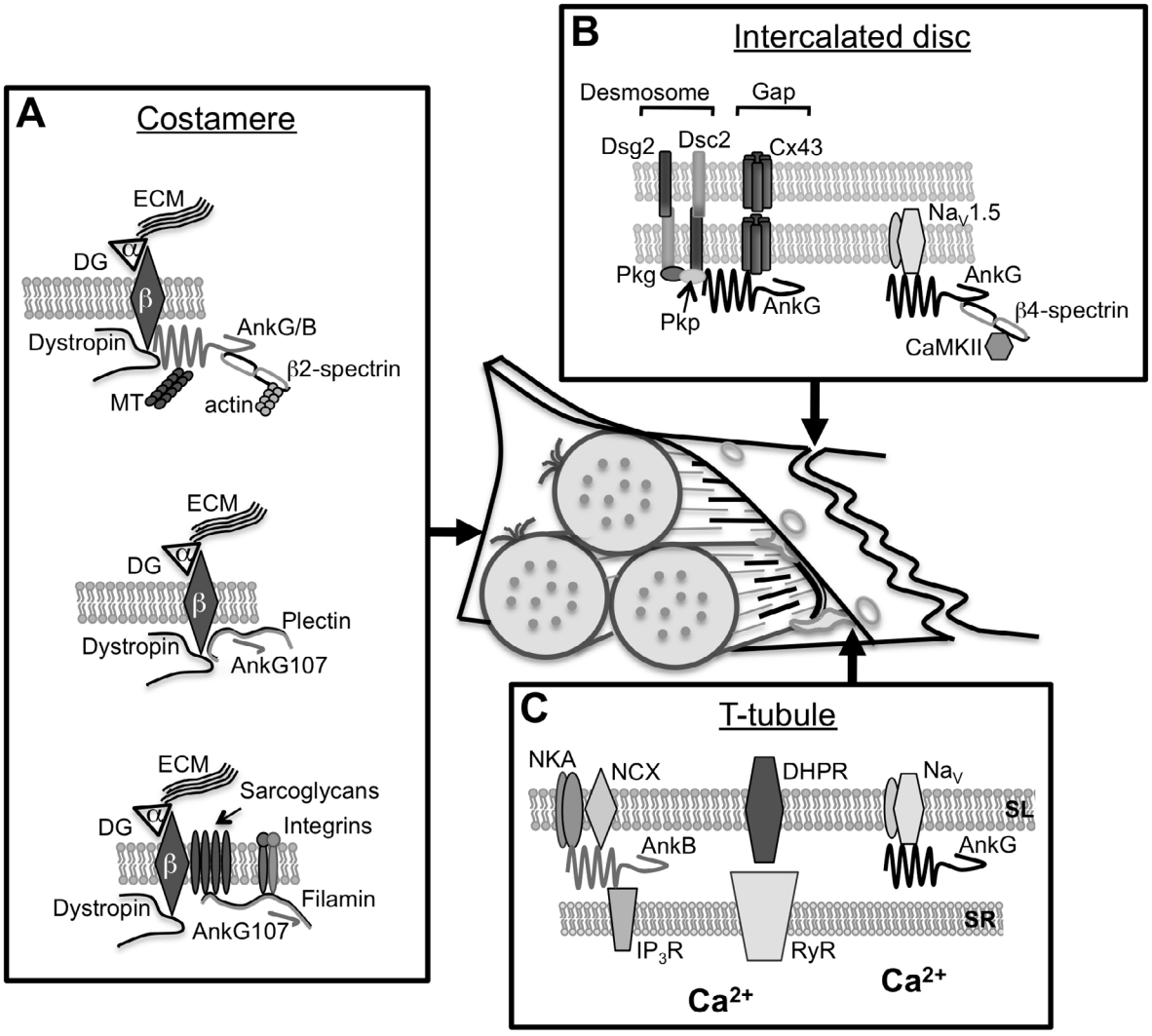

Figure 2: *Ankyrin membrane domain specialization in myocytes*. (A) Costameres: an interaction between ankyrin-B and β2-spectrin recruits and retains dystrophin, β-dystroglycan (DG), and microtubules to the sarcolemma. The retention of dystrophin and β-dystroglycan specifically at costameres is dependent on direct interactions with ankyrin-G. Truncated ankyrin-G isoforms (AnkG107) are also expressed at costameres through interactions with plectin and filamin. (B) Intercalated disc: Ankyrin-G interacts with components of the gap junction (connexin43, Cx43) and desmosomal complex (plakophilin-2, Pkp). Other desmosomal components: desmoglein-2 (Dsg2), desmocollin-2 (Dsc2), and plakoglobin (Pkg). Ankyrin-G targets and scaffolds $Na_V1.5$ at the ICD where it forms a local signaling complex with β4-spectrin and Ca^{2+}/calmodulin kinase II (CaMKII). (C) T-tubule: Ankyrin-B targets and retains the sodium/calcium exchanger (NCX), sodium/potassium pump (NKA), and inositol triphosphate receptor (IP_3R) at T-tubules of ventricular myocytes. The functional coupling of the sarcolemmal (SL) dihydropyridine receptor (DHPR) and ryanodine receptor (RyR) in the sarcoplasmic reticulum (SR) propagates calcium-induced calcium release. Ankyrin-G retains a subpopulation of voltage-gated sodium channels (Na_V) at the T-tubules.

$Na_V1.5$ localization in lateral membranes, reduced sodium current density, and attenuated impulse propagation [80,81]. Interestingly, the ICD-resident protein SAP97 also interacts with $Na_V1.5$ via its last three amino acids. Inhibiting this interaction reduces $Na_V1.5$ protein expression and localization at pseudo-ICDs, in addition to reducing total sodium current density [80]. An unresolved question about the differential localization of $Na_V1.5$ is what regulates channel interaction with syntrophin or SAP97 given that they both share the same binding site on $Na_V1.5$.

In ventricular myocytes, ankyrin-G is required for the targeting and retention of $Na_V1.5$ at intercalated discs. Ankyrin-G directly binds to $Na_V1.5$ via a conserved ankyrin-binding domain present in the cytoplasmic loop between the DII and DIII homologous domains [15]. Disruption of this interaction causes the loss of $Na_V1.5$ membrane expression at ICDs and reduced sodium current density [15,42]. Ankyrin-G-dependent enrichment of $Na_V1.5$ at the ICD is important for action potential propagation between adjoining myocytes [82]. Interestingly, a $Na_V1.5$ missense mutation that disrupted ankyrin-G binding and reduced channel membrane localization was linked to a case of Brugada syndrome, which is a cardiac arrhythmia characterized by ventricular conduction abnormalities and reduced $Na_V1.5$ function [15]. Phosphorylation also regulates $Na_V1.5$ channel activity and in the costamere β4-spectrin is a novel calcium/calmodulin-dependent protein kinase II (CaMKII) binding protein [83]. A direct interaction between ankyrin-G and β4-spectrin retains CaMKII in close proximity to $Na_V1.5$ [83]. Channel phosphorylation by CaMKII enhances the peak sodium current and changes the channel's inactivation gating

[83]. The unique mechanoelectrical properties of the intercalated disc are dependent in part on ankyrin-G targeting and scaffolding of plakophilin-2, connexin43, and $Na_V1.5$.

Costameres: Costameres are submembranous protein complexes that facilitate the lateral transmission of contractile force to the sarcolemma, surrounding extracellular matrix, and neighboring myocytes. They overlie the Z-lines, which define the boundaries of an individual sarcomere, and facilitate mechanotransduction through the actions of focal adhesion proteins such as vinculin, α-actinin, and β1 integrin. Also residing in the costamere, the dystrophin-glycoprotein complex (DGC) connects the myocyte cytoskeleton through the sarcolemma to the surrounding extracellular matrix thereby providing structural integrity for the sarcolemmal membrane. While several transmembrane and peripheral proteins contribute to DGC stability and sarcolemmal integrity, both dystrophin and dystroglycan play central roles in this complex. Dystroglycan is proteolytically processed into an extracellular α-subunit and transmembrane-spanning β-subunit. The link between the extracellular matrix (ECM) and the cytoskeleton is mediated by β-dystroglycan by binding to the ECM-associated α-dystroglycan and dystrophin, which binds to actin and intermediate filaments.

Both ankyrin-B and ankyrin-G have been associated with the recruitment and retention of DGC components to the costamere. Specifically, ankyrin-B and ankyrin-G bind to dystrophin, while ankyrin-G binds to β-dystroglycan [35] (Figure 2). The sarcolemmal recruitment/ retention of dystrophin by ankyrin-B is dependent on ankyrin-B interaction with dynactin-4, a component of the dynactin complex that links membrane cargo to the submembranous actin filaments. In skeletal muscle, the loss of ankyrin-B or its intermediary dynactin-4 results in decreased sarcolemmal localization of dystrophin, β-dystroglycan, and costamere-associated microtubules [8]. Not surprisingly, ankyrin-B haploinsufficient mice display greater muscle damage following exercise compared to their wild-type littermates [8]. While ankyrin-B regulates the sarcolemmal localization of dystrophin and β-dystroglycan, ankyrin-G is important for the retention of these proteins at the costamere [35]. In skeletal muscle, the loss of ankyrin-G reduces the costameric localization of both dystrophin and β-dystroglycan, while their sarcolemmal localization remains intact [35]. Based on these findings, it has been suggested that dystrophin, β-dystroglycan and a subset of microtubules are initially recruited/ retained at the sarcolemma by an ankyrin-B/dynactin-4 protein complex and the further refinement of their localization to costameres is facilitated by an ankyrin-G/β2-spectrin protein complex. While this is a very tentative model, unresolved issues include the relationship between ankyrin-G and ankyrin-B at the costameres, the function of DGC adaptor proteins such as syntrophins in this protein complex, and the characterization of different ankyrin isoforms at the costameres.

Many different ankyrin-G isoforms are expressed in striated muscle due to alternative splicing. In addition to the full-length ankyrin-G isoform with all three functional domains, truncated isoforms lacking the membrane-binding domain have been detected in skeletal and cardiac tissue. Interestingly, these truncated isoforms include a novel stretch of 76 amino acids in the C-terminal domain that have been previously shown to mediate interactions with obscurin, a large structural protein implicated in myofibrillogenesis and predominantly localized to the sarcomere M-line [84]. In addition to obscurin, two actin-binding scaffolding proteins plectin and filamin that localize to the costamere interact with this 76 amino acid domain [85]. In the costamere, plectin and filamen act as adaptor/scaffolding proteins that interact with components of the DGC and the β-integrin complex (Figure 2). While the interactions with plectin and filamin most likely contribute to the stability of these truncated ankyrin-G isoforms at costameres, the costameric functions of these isoforms remain to be determined. In addition, the relationship between these truncated isoforms and the full-length version is another unresolved issue.

Conclusions

Since the initial discovery of the ankyrin/β-spectrin cytoskeletal complex some 35 years ago, there has been a tremendous growth in our understanding of how this complex functions in both normal and diseased states. Historically, ankyrin dysfunction was only associated with haemolytic anemia, but now dysfunction of ankyrin and associated proteins has been connected to numerous cardiac arrhythmias, epilepsy, bipolar disorder, and a type of neonatal diabetes. The vast majority of our knowledge about ankyrin biology has come from analysis and interpretation of ankyrin and β-spectrin function at the plasma membrane in a static situation. While this analysis has been tremendously productive, it doesn't provide a complete view of the entirety of ankyrin/ β-spectrin functions. For example, fundamental unresolved questions include what regulates ankyrin specificity for membrane proteins, where along the biosynthetic pathway does ankyrin interact with membrane proteins, and how does the ankyrin/β-spectrin complex orchestrate differential targeting of membrane proteins. Given that both ankyrin and β-spectrin are involved with elemental biological processes such as establishing subcellular polarity, maintaining membrane excitability, and reinforcing adhesive junctions, it will come as no surprise if they are implicated in the molecular pathogenesis of many more diseases.

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