Membrane Domains Based on Ankyrin and Spectrin Associated with Cell–Cell Interactions

Vann Bennett and Jane Healy

Howard Hughes Medical Institute, and Departments of Cell Biology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Correspondence: v.bennett@cellbio.duke.edu

Nodes of Ranvier and axon initial segments of myelinated nerves, sites of cell–cell contact in early embryos and epithelial cells, and neuromuscular junctions of skeletal muscle all perform physiological functions that depend on clustering of functionally related but structurally diverse ion transporters and cell adhesion molecules within microdomains of the plasma membrane. These specialized cell surface domains appeared at different times in metazoan evolution, involve a variety of cell types, and are populated by distinct membrane-spanning proteins. Nevertheless, recent work has shown that these domains all share on their cytoplasmic surfaces a membrane skeleton comprised of members of the ankyrin and spectrin families. This review will summarize basic features of ankyrins and spectrins, and will discuss emerging evidence that these proteins are key players in a conserved mechanism responsible for assembly and maintenance of physiologically important domains on the surfaces of diverse cells.

Spectrins are flexible rods 0.2 microns in length with actin-binding sites at each end (Shotton et al. 1979; Bennett et al. 1982) (Fig. 1A). Spectrins are assembled from α and β subunits, each comprised primarily of multiple copies of a 106-amino acid repeat (Speicher and Marchesi 1984). In addition to the canonical 106-residue repeat, β spectrins also have a carboxy-terminal pleckstrin homology domain (Zhang et al. 1995; Macias et al. 1994) and tandem amino-terminal calponin homology domains (Bañuelos et al. 1998), whereas α spectrins contain an Src homology domain 3 (SH3) site (Musacchio et al. 1992), a calmodulin-binding site (Simonovic et al. 2006), and EF hands (Travé et al. 1995) (Fig. 1A). Spectrin α and β subunits are assembled antiparallel and side-to-side into heterodimers, which in turn are associated head-to-head to form tetramers (Clarke 1971; Shotton et al. 1979; Davis and Bennett 1983) (Fig. 1A). In human erythrocytes, in which spectrin was first characterized (Marchesi and Steers 1968; Clarke 1971), actin oligomers containing 10–14 monomers are each linked to five to six spectrin tetramers by accessory proteins to form a geodesic domelike structure that has been resolved by electron microscopy (Byers and Branton 1985). The principal proteins at the spectrin–actin junction are protein 4.1,
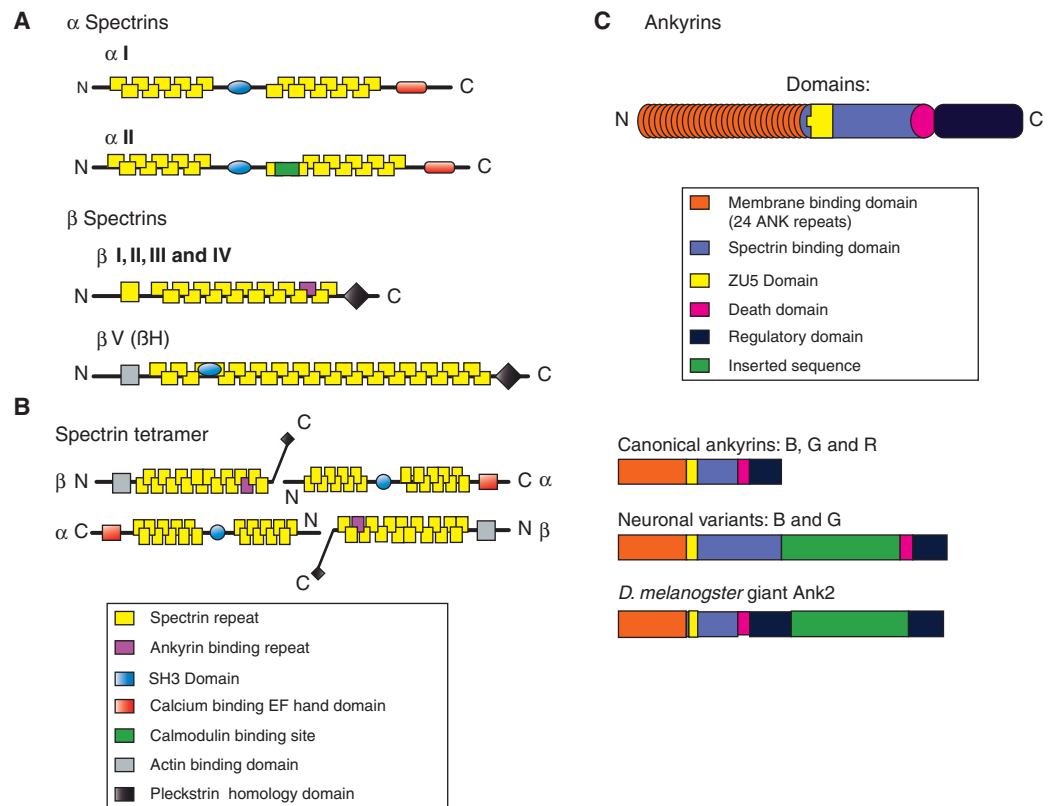
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Figure 1. Domain structure and variants of spectrin and ankyrin proteins. (*A*) Molecular domains of spectrins: Two α spectrins and five β spectrins are shown. Spectrins are comprised of modular units called spectrin repeats (yellow). Other domains such as the ankyrin binding domain (purple), Src-homology domain 3 (SH3, blue), EF-hand domain (red), and calmodulin-binding domain (green) promote interactions with binding targets important for spectrin function. The pleckstrin homology domain (black) promotes association with the plasma membrane and the actin binding domain (grey) tethers the spectrin-based membrane skeleton to the underlying actin cytoskeleton. (*B*) The spectrin tetramer, the fundamental unit of the spectrin-based membrane skeleton. The spectrin repeat domains of α and β spectrin associate end-to-end to form heterodimers. Heterodimers associate laterally in an antiparallel fashion to form tetramers. The tetramers can then associate end-to-end to form extended macromolecules that link into a geodesic dome shape directly underneath the plasma membrane. (*C*) Molecular domains present in canonical ankyrins. The membrane binding domain of ankyrin isoforms (orange) is comprised of 24 ANK repeats. The spectrin binding domain (green-blue) allows ankyrins to coordinate integral membrane proteins to the membrane skeleton. The death domain (pink) is the most highly conserved domain. The regulatory domain (brown) is the most variable region of ankyrins. The regulatory domain interacts intramolecularly with the membrane binding domain to modulate ankyrin's affinity for other binding partners. All ankyrins and spectrins are subject to alternative splicing, which further increases their functional diversity.

adducin, tropomyosin, tropomodulin, and dematin (Bennett and Baines 2001) (Table 1).

Spectrin is coupled to the inner surface of the erythrocyte membrane primarily through association with ankyrin, which is in turn linked to the cytoplasmic domains of the anion exchanger (Bennett 1978; Bennett and Stenbuck 1979a,b) and Rh/RhAG ammonium transporter (Nicolas et al. 2003). The spectrin-based membrane skeleton and its connections through ankyrin to membrane-spanning proteins are essential for survival of erythrocytes in the circulation, and mutations in these proteins result in hereditary hemolytic anemia

Table 1. Binding partners of spectrin and ankyrins

Spectrin Binding Partners			
Alpha	**Beta**		
Transporters/ion channels	*Membrane anchors*		
EnNaC (sodium)	PI lipids		
NHE2 (ammonium)	Band 4.1		
	Ankyrin		
	EAAT4 (glutamate)		
Membrane receptors	*Signaling*		
NMDA receptor	RACK-1		
Signaling	*Cytoskeleton/cellular transport*		
HsSH3pb1	F-actin		
Calmodulin	Adducin		
	Dynactin		
Ankyrin Binding Partners			
Membrane BD	**Spectrin BD**	**DD**	**REG D**
Ion channels:	Spectrin	FasL	Hsp40
Anion exchanger			Obscurin
Na+/K+ATPase			PP2A
Voltage-gated			
Na+ channels			
Na+/Ca^{2+} Exchanger			
KCNG2/3			
Rh antigen			
IP3 receptor			
Ryanodine receptor			
Cell adhesion molecules:			
L1-CAMs			
CD44			
E-cadherin			
Dystroglycan			
Cellular transport:			
Tubulin			
Clathrin			

(Bennett and Healy 2008). The ankyrin-binding sites of β spectrins 1–4 are located in the 15th spectrin repeat, which is folded identically to other repeats but has distinct surface-exposed residues (Davis et al. 2008; Ipsaro et al. 2009; Stabach et al. 2009) (Figs. 1A, 2A). Mammalian β-5 spectrin and its ortholog β-H spectrin in *Drosophila* and *Caenorhabditis elegans* are the only β spectrins lacking ankyrin-binding activity (Dubreuil et al. 1990; Thomas et al. 1998; McKeown et al. 1998; Stabach and Morrow 2000).

Ankyrin interacts with β spectrins through a ZU5 domain (Mohler et al. 2004a; Kizhatil et al. 2007a; Ipsaro et al. 2009) (Fig. 1B), and

with most of its membrane partners through ANK repeats (Bennett and Baines 2001) (Fig. 2C,D). In addition, ankyrins have a highly conserve "death domain" and a carboxy-terminal regulatory domain (see the following discussion). The 24 ANK repeats are stacked in a superhelical array to form a solenoid (Michaely et al. 2002). Interestingly, the ANK repeat stack behaves like a reversible spring when stretched by atomic force microscopy, and may function in mechano-coupling in tissues such as the heart (Lee et al. 2006). ANK repeats are components of many proteins and participate in highly diverse protein interactions (Mosavi et al. 2004) (Fig. 2C).
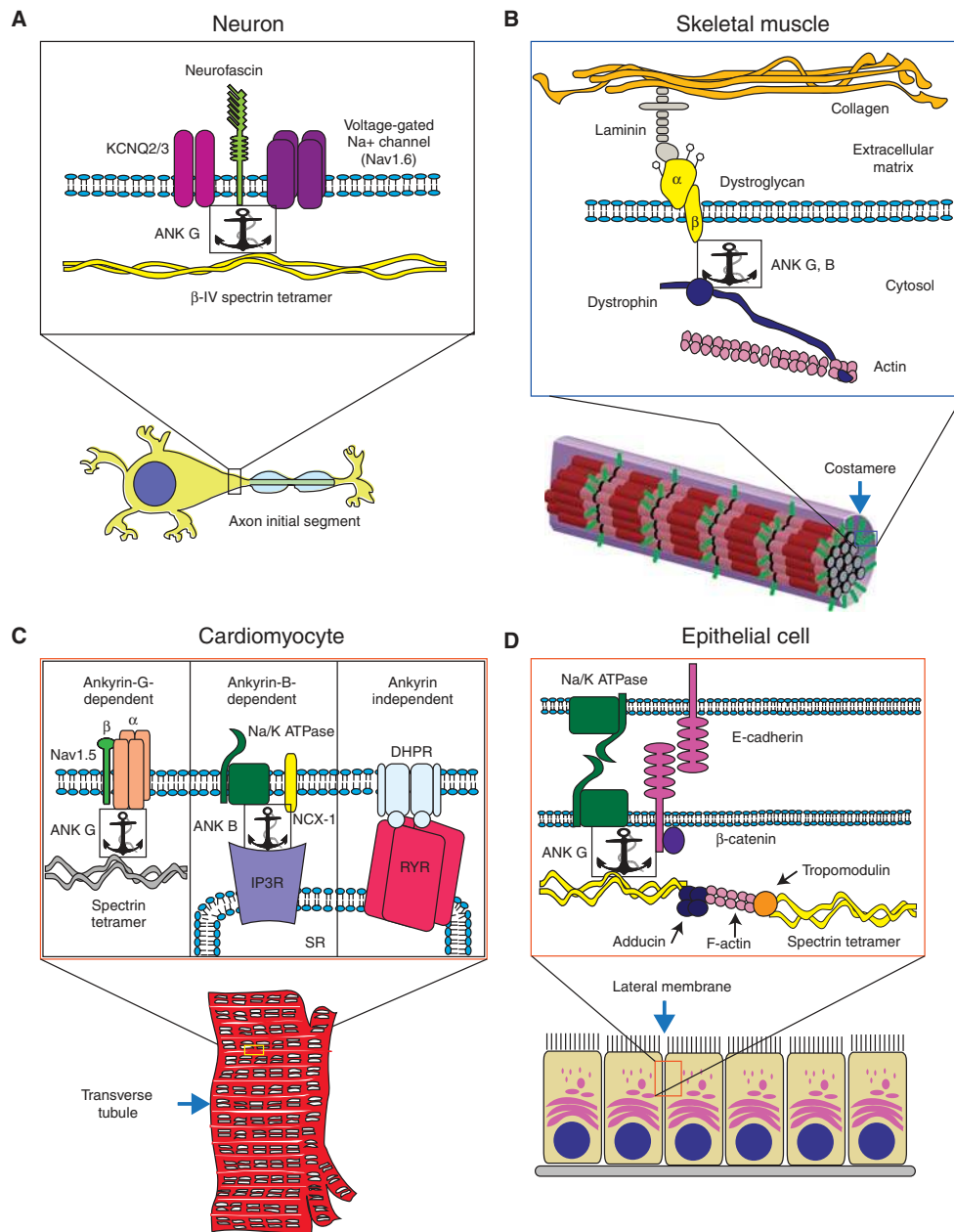
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Figure 2. Ankyrins and spectrins organize macromolecular complexes in diverse types of specialized membranes. (*A*) Ankyrin-G forms a complex with β-IV spectrin, neurofascin (a cell adhesion protein), and ion channels (KCNQ2/3 and voltage-gated sodium channel) at axon initial segments in Purkinje neurons. (*B*) In force buffering costameres of skeletal muscle, ankyrins -B and -G cooperate to target and stabilize key components of the dystroglycoprotein complex. At the membrane, ankyrin-G binds to dystrophin and β-dystroglycan. (*C*) In cardiomyocyte transverse tubules, ankyrins -B and -G coordinate separate microdomains. Ankyrin-B binds $Na+/K+$ ATPase, $Na+/Ca^{2+}$ exchanger (NCX-1), and the inositol triphosphate receptor (IP3R). Ankyrin-G forms a complex with Nav1.5 and spectrin. (*D*) Ankyrin-G in epithelial lateral membrane assembly. Ankyrin-G binds to E-cadherin, β-2 spectrin, and the $Na+/K+$ ATPase. Spectrins are connected via F-actin bridges bound to α/γ adducin and tropomodulin.

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This versatile motif currently is being exploited using designed ANK repeat proteins (DARPins) engineered to interact with specific ligands that can function as substitutes for antibodies (Stumpp and Amstutz 2007; Steiner et al. 2008).

Spectrin and ankyrin family members are expressed in most, if not all, animal (metazoan) cells, but are not present in bacteria, plants, or fungi. Spectrins are believed to have evolved from an ancestral α-actinin containing calponin homology domains and two spectrin repeats but not other domains (Thomas et al. 1997; Pascual et al. 1997). Ankyrin repeats are expressed in all phyla, presumably because of a combination of evolutionary relationships and in cases of bacteria and viruses by horizontal gene transfer. However, the spectrin-binding domain of ankyrin is present only in metazoans (Fig 1B). It is possible that evolution of ankyrins and spectrins could have been one of the adaptations required for organization of cells into tissues in multicellular animals.

The human spectrin family includes two α subunits and five β subunits, whereas *Drosophila* and *C. elegans* have a single α subunit and two β subunits (Bennett and Baines 2001). Vertebrate ankyrins are encoded by three genes: ankyrin-R (ANK1) (the isoform first characterized in erythrocytes and also present in a restricted distribution in brain and muscle), ankyrin-B (ANK2), and ankyrin-G (ANK3). Vertebrate ankyrins evolved from a single gene in early chordates (Cai and Zhang 2006). *C. elegans* ankyrin is encoded by a single gene termed *unc-44* (Otsuka et al. 1995), whereas the *Drosophila* genome contains two ankyrin genes: *ankyrin* (Dubreuil and Yu 1994) and *ankyrin2* (Bouley et al. 2000).

Mammalian ankyrins -B and -G are co-expressed in most cells, although they have distinct functions (Mohler et al. 2002; Abdi et al. 2006). Ankyrins -B and -G are closely related in their ANK repeats, and spectrin-binding domains, but diverge in their carboxy-terminal regulatory domains. Regulatory domains are natively unstructured and extended (Abdi et al. 2006). These flexible domains engage in intramolecular interactions with the membrane-binding and spectrin-binding domains (Hall and Bennett 1987; Davis et al. 1992; Abdi et al. 2006) that modulate protein associations and provide functional diversity between otherwise conserved ankyrins.

In addition to the standard versions of ankyrins and spectrin subunits depicted in Figure 1, many variants of these proteins are expressed with the addition and/or deletion of functional domains because of alternative splicing of pre-mRNAs. For example, β spectrins can lack PH domains (Hayes et al. 2000), and giant ankyrins have insertions of up to 2000 residues (Kordeli et al. 1995; Chan et al. 1993; Pielage et al. 2008; Koch et al. 2008), whereas other ankyrins lack either the entire membrane-binding domain (Hoock et al. 1997), or both membrane- and spectrin-binding domains (Zhou et al. 1997). The insertions in 440 kDa ankyrin-B and 480 kDa ankyrin-G (Fig. 1B) have an extended conformation that potentially could have specialized roles in connections between the plasma membrane and cytoskeleton of axons where these giant ankyrins reside (Chan et al. 1993; Kordeli et al. 1995) (Fig. 1B). Interestingly, the inserted sequences in *Drosophila* giant ankyrins interact with microtubules at the presynaptic neuromuscular junction (Pielage et al. 2008) (see the following section).

MEMBRANE-SPANNING PROTEIN PARTNERS

Diverse families of membrane-spanning proteins, including ion channels, pumps, and exchangers as well as cell adhesion molecules have independently acquired ankyrin-binding activity multiple times in metazoan evolution (Table 1). These proteins include the anion exchanger (AE1) (Bennett and Stenbuck 1979b; Stefanovic et al. 2007), voltage-gated sodium channels (Srinivasan et al. 1988; Garrido et al. 2003; Lemaillet et al. 2003), Na/K ATPase (Nelson and Veshnock 1987), Na/Ca exchanger (Li et al. 1993; Mohler et al. 2005; Cunha et al. 2007), IP3 receptors (Mohler et al. 2003; Mohler et al. 2005; Kline et al. 2008), Rh ammonium transporter (Nicolas et al. 2003; Lopez et al. 2005; Sohet

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et al. 2008), KCNQ2/3 channels (Pan et al. 2006; Chung et al. 2006; Rasmussen et al. 2007), Kv3.1 channels (Devaux et al. 2003; Xu et al. 2007), and cell adhesion molecules including L1 CAMs (Davis and Bennett 1994; Zhang et al. 1998), CD44 (Bourguignon et al. 1992), E- and N-cadherin (Kizhatil et al. 2007a), and β-dystroglycan (Ayalon et al. 2008) (Table 1). The ankyrin-binding sites have been defined in many of these proteins as relatively short stretches of 10–20 amino acids that do not contain a single defining motif (Fig. 3A). However, once ankyrin-binding activity appears in a protein family, the binding sites remain highly conserved. For example, the ankyrin-binding sites of human L1CAMs are nearly identical to the binding site of the L1 homolog of *C. elegans* (Chen et al. 2001), whereas sites of voltage-gated sodium channels and KCNQ2/3 channels are absent in *Drosophila* and but are conserved between humans and zebra fish (Pan et al. 2006).

Some membrane proteins such as NMDA receptors (Wechsler and Teichberg 1998), the neuronal glutamate transporter EAAT4 (Jackson et al. 2001), and the epithelial sodium channel (EnaC) (Rotin et al. 1994; Zuckerman et al. 1999) can associate directly with spectrin independently of ankyrin. Spectrin has also been reported to coimmunoprecipitate with the presynaptic voltage-sensitive calcium channel (Sunderland et al. 2000), although a direct interaction between these proteins has not been shown. Together, ankyrins and spectrins display a remarkable capacity for interactions with physiologically important membrane proteins.

AXON INITIAL SEGMENTS AND NODES OF RANVIER

Axon initial segments (Fig. 2A) and nodes of Ranvier are highly enriched in 480/270 kDa alternatively spliced variants of ankyrin-G (Kordeli et al. 1995), β-4 spectrin (Berghs et al. 2000; Lacas-Gervais et al. 2004), as well as ankyrin-binding proteins including voltage-gated Na channels and 186 kDa neurofascin (Davis et al. 1996). Axon initial segments are of special importance as the integrator sites of neurons in which inputs from dendritic synapses (sometimes on the order of several hundred thousand) are transduced into action potentials. These action potentials result in both signaling to other neurons or target cells when propagated down the axon, and to modulation of synaptic function when back-propagated into dendritic shafts (Waters et al. 2005). In myelinated axons, action potentials are propagated at periodic interruptions in the myelin sheathe known as nodes of Ranvier.

A

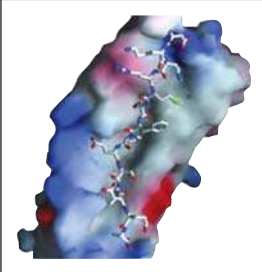| Ankyrin Binding Site	
NaV	VPIAGESDFE
KCNQ2	PYIAEGESDTDSD
E-cadherin	KEPLLPPEDDTRDNVYY-YDEEGGGEED
Neurofascin/L1CAMs	QFNEDGSFIGQY
RhBG	KLPFLDSPP
Dystroglycan	GVPIIFAD ELDDSK
AE1	PAVLTRSGDPS

B

Figure 3. Ankyrins bind to natively unstructured regions of many proteins. (*A*) Known binding sites of ankyrin proteins. All of these regions lie in regions predicted to be intrinsically unstructured (http://iupred.enzim.hu/IUPs.html). Abbreviations: NaV, voltage-gated sodium channels; KCNQ2, voltage-gated potassium channels; RhBG, rhesus blood group antigen; AE1, anion exchanger. (*B*) A theoretical model of how the ankyrin membrane binding domain could bind to unstructured peptides. This pocket is 240 angstroms in length with a variety of surface exposed residues.

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Myelin and nodes of Ranvier are adaptations of vertebrates that allow rapid nerve conduction using small caliber axons.

Targeted knockout of ankyrin-G in the postnatal cerebellum in mice results in severe ataxia, and loss of ability to fire action potentials as well as clustering of voltage-gated Na channels (Nav1.6) at axon initial segments of Purkinje neurons (Zhou et al. 1998; Jenkins and Bennett 2001). In addition, β-4 spectrin is absent and neurofascin is no longer restricted to ankyrin-G-deficient initial segments (Jenkins and Bennett 2001). These result in Purkinje neurons that have been recapitulated in cultured hippocampal neurons, where knockdown of ankyrin-G results in mis-localization of β-4 spectrin, voltage-gated sodium channels, as well as neurofascin (Yang et al. 2007; Hedstrom et al. 2007).

β-4 spectrin knockout mice show diminution of the membrane undercoat and increased membrane blebbing at nodes of Ranvier (Lacas-Gervais et al. 2004). Ankyrin-G recruits β-4 spectrin to nodes and initial segments (Jenkins and Bennett 2001; Yang et al. 2007), and is still present in the absence of β-4 spectrin. β-4 spectrin thus plays an important role in stabilizing these excitable membranes, following establishment by ankyrin-G alone at initial segments or by ankyrin-G in collaboration with axonal neurofascin and Schwann cell gliomedin at nodes of Ranvier (Eshed et al. 2005; Dzhashiashvili et al. 2007).

KCNQ2/3(Kv7) channels modulate activity of voltage-gated Na channels, and mutations in these channels results in hyperexcitability phenotypes including epilepsy (Maljevic et al. 2008; Neubauer et al. 2008). KCNQ2/3 channels colocalize with voltage-gated Na channels at nodes of Ranvier and axon initial segments (Devaux et al. 2004; Pan et al. 2006; Rasmussen et al. 2007). KCNQ2/3 channels have ankyrin-binding sites in their cytoplasmic domains, and require ankyrin-G for targeting to axon initial segments (Pan et al. 2006; Chung et al. 2006; Rasmussen et al. 2007).

Strikingly, knockdown of ankyrin-G in cultured hippocampal neurons results in loss of voltage-gated Na channels from initial segments and conversion of these domains into dendrites (Hedstrom et al. 2008). Ankyrin-G thus is required for preservation of the entire axon initial segment. It will be of interest to determine the role of ankyrin-G in the establishment of initial segments as well as whether ankyrin-G operates downstream of axonal polarity pathways.

Axon initial segments receive direct input through axo-axonic synapses of interneurons, which modulate neuronal output and may have roles in diseases such as epilepsy and schizophrenia (Howard et al. 2005). Loss of the neurofascin enrichment at the initial segment in ankyrin-G-deficient Purkinje neurons results in disruption of a class of synapses formed by interneurons that interconnect Purkinje neurons in the cerebellum (Ango et al. 2004). Ankyrin-G thus is responsible for stabilizing transcellular connections as well as organizing the composition of initial segments.

Spectrin and ankyrin are likely to function in other axonal domains. For example, paranodes are specialized zones immediately adjacent to the nodal gap in myelinated axons that contain shaker-type potassium channels and are characterized ultrastructurally by prominent axo-glial junctions. Paranodes contain a membrane skeleton distinct from nodes that includes β-2 spectrin, ankyrin-B, and protein 4.1 B (Ogawa et al. 2006). α-2 Spectrin is present in paranodes where it associates with β-2 spectrin, and also at the nodal gap where it partners with β-4 spectrin. α-2 Spectrin mutations in zebra fish result in abnormal development of nodes of Ranvier, and are responsible for stabilizing initial clusters of voltage-gated Na channels (Voas et al. 2007).

Ankyrin-binding activity of its membrane partners is not "hardwired," but is subject to regulation. Phosphorylation of neurofascin at the FIGQY tyrosine in its ankyrin-binding site abolishes ankyrin-binding (Garver et al. 1997; Whittard et al. 2006) and results in gain of function in binding to doublecortin (Kizhatil et al. 2002). FIGQY-phosphorylated neurofascin is excluded from the node of Ranvier but is concentrated in paranodes (Jenkins et al. 2001). Ankyrin-binding activity of the

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voltage-gated sodium channel is markedly enhanced by phosphorylation by casein kinase 2 (Bréchet et al. 2008). Interestingly, casein kinase 2 is concentrated at nodes of Ranvier and axon initial segments, and could provide a local activation signal for stabilizing voltage-gated sodium channels at these sites (Bréchet et al. 2008).

NEUROMUSCULAR JUNCTIONS

Spectrin and ankyrin stabilize neuromuscular junctions through both presynaptic and postsynaptic mechanisms. Presynaptic β spectrin is required for normal transmitter release and to stabilize *Drosophila* neuromuscular junctions following initial establishment of these synapses (Featherstone et al. 2001; Pielage et al. 2005). Interestingly, two groups independently identified presynaptic giant ankyrin-2 isoforms through different unbiased forward genetic screens for mutations affecting the *Drosophila* neuromuscular junction (Koch et al. 2008; Pielage et al. 2008). The phenotypes of ankyrin-mutant junctions included retraction of synaptic boutons, loss of axonal microtubules, and misorganization of synaptic cell adhesion molecules. The defects in synaptic stability increased with distance from the neuron cell body and were accompanied by accumulation of synaptic vesicles in axons, suggesting a role of ankyrins in axonal transport in addition to their local function at the synapse (Koch et al. 2008).

Spectrin and ankyrin-G are localized in specialized postsynaptic domains in mammalian neuromuscular junctions that are distinct from the acetylcholine receptor and are enriched in voltage-gated sodium channels (Flucher and Daniels 1989; Wood and Slater 1998; Kordeli et al. 1998; Bailey et al. 2003). Ankyrin-B also is located at the periphery of mammalian neuromuscular junctions (Ayalon et al. 2008). Experiments in flies and mice show that postsynaptic spectrin and ankyrins are required to stabilize neuromuscular junctions. Knockdown of postsynaptic spectrin in the *Drosophila* by siRNA results in misorganization of active zones and abnormal growth of the neuromuscular junction (Pielage et al. 2006). Similarly, knockdown of postsynaptic ankyrin-B in adult mouse muscle results in shrinkage of the neuromuscular junction from its adult form back to the size of neonatal junctions (Ayalon et al. 2008).

COSTAMERES

Costameres (Fig. 2B) are specialized domains formed at the junction of the plasma membrane and Z-discs of peripheral myofibrils in skeletal muscle and cardiomyocytes (Pardo et al. 1983; Rybakova et al. 2000; Ervasti 2003; Bloch et al. 2004). Costameres function as force buffers that transmit force across the plasma membrane from sarcomeres to the extracellular matrix, and protect the muscle plasma membrane from damage during contraction. The dystrophin-glycoprotein complex (DGC) provides a transmembrane connection at costameres through association of dystrophin with γ-actin and dystroglycan, and dystroglycan with extracellular laminin (Ervasti, 2003; Rybakova et al. 2000). The dystrophin-glycoprotein-complex is absent from the plasma membrane in Duchenne muscular dystrophy, which results in membrane damage and contributes to death of muscle cells (Ervasti et al. 1990; Cohn and Campbell 2000; Dalkilic and Kunkel 2003).

Ankyrin-B and ankyrin-G cooperate in localization of dystrophin and β-dystroglycan at costameres and are required to prevent exercise-induced injury (Ayalon et al. 2008). Ankyrin-B is required for transport of β-dystroglycan to the plasma membrane, whereas ankyrin-G retains β-dystroglycan at costameres. Loss of ankyrin-B in skeletal muscle is accompanied by loss of microtubules, both at the neuromuscular junction (see previous discussion) as well as costameres (Ayalon et al. 2008). Ankyrin-B binds directly to dynactin-4/p62 of the dynactin complex, and may capture fast-growing ends of microtubules at costameres and neuromuscular junctions to establish transport routes from the trans-Golgi network for newly synthesized β-dystroglycan (Ayalon et al. 2008).

CARDIOMYOCYTE T-TUBULE MICRODOMAINS

Rhythmic contraction of mammalian hearts requires nearly synchronous waves of calcium release and reuptake throughout the intracellular space of ventricular cardiomyocytes. This is achieved though precise placement of membrane transporters and signaling molecules related to import and export of calcium within microdomains of T-tubules (Fig. 2C). T-tubules are invaginations of the plasma membranes that form a complex three-dimensional network juxtaposed to the sarcoplasmic reticulum (Brette and Orchard 2007). Ventricular myocyte T-tubules contain three microdomains that can be resolved by high resolution light microscopy: A domain containing L-type voltage-gated calcium channels complexed with ryanodine receptors in the sarcoplasmic reticulum, a second domain enriched in the Na/Ca exchanger, and a third domain enriched in voltage-gated sodium channels (Scriven et al. 2000). Voltage-gated sodium channels are responsible for activating voltage-gated calcium channels, which admit a small amount of calcium and activate calcium release from the sarcoplasmic reticulum. The Na/Ca exchanger contributes to calcium homeostasis by export of calcium back across the T-tubule. Evidence will be reviewed below indicating that ankyrin-B is required for the Na/Ca exchanger microdomain, and ankyrin-G is required for the domain enriched in voltage-gated sodium channels.

The Na/Ca exhanger (NCX1) binds to ankyrin-B and colocalizes with ankyrin-B in cardiomyocyte T-tubules (Li et al. 1993; Mohler et al. 2005; Cunha et al. 2007). In addition, NCX1 and ankyrin-B also colocalize with the Na/K ATPase in the T-tubule membrane and with the IP3 receptor in the sarcoplasmic reticulum (Mohler et al. 2005). Haploinsufficiency of ankyrin-B in mice results in selective loss of T-tubule-localized sodium/calcium exchanger, Na/K ATPase as well as IP3 receptor from adult cardiomyocytes (Mohler et al. 2003; 2005). Moreover, ankyrin-B-deficient cardiomyocytes show increased contractility and increased calcium transients (Mohler et al. 2003; 2005).

These observations suggest that ankyrin-B-dependent colocalization of NCX1 and Na/K ATPase could result in functional coupling between these transporters, with Na/K ATPase-driven export of sodium ions entering the cell in exchange for calcium ions. The IP3 receptor in the ankyrin-B complex has been proposed to function in coupled calcium export from the sarcoplasmic reticulum directly through NCX1 (Mohler et al. 2005). The physiological importance of the ankyrin-B-microdomain in T-tubules is supported by findings that ankyrin-B-deficient mice and humans heterozygous for loss-of-function mutations of ankyrin-B show stress-induced sudden cardiac death and cardiac arrhythmia symptoms (Mohler et al. 2003; 2004b).

Nav1.5 and ankyrin-G and are both localized to T-tubules and intercalated discs of adult cardiomyoctes (Mohler et al. 2004c). Although high-resolution double-labeling for these proteins has not been reported, several findings strongly indicate that Nav1.5 and ankyrin-G are molecular partners in the heart. E1053K mutation of the ankyrin-binding site of Nav1.5 eliminates ankyrin-G-binding as well as ability of Nav1.5 to accumulate at the cell surface of cardiomyocytes (Mohler et al. 2004c). Interestingly, this Nav1.5 mutation is associated with Brugada syndrome, which is a cardiac arrhythmia associated with loss-of-function mutations of Nav1.5. Nav1.5 requires interaction with ankyrin-G through ank repeats 14 and 15 for expression at the cell surface of neonatal cardiomyocytes (Lowe et al. 2008).

EPITHELIAL LATERAL MEMBRANES

The lateral membrane domain of epithelial cells (Fig. 2D) is of considerable physiological importance because of its roles in salt and water homeostasis and protection of epithelial tissues from mechanical stress. Moreover, loss of this specialized domain is a hallmark of metastatic cancer cells. Early immunofluorescence studies identified ankyrin and spectrin in a polarized pattern localized to lateral membranes of cultured epithelial cells as well as in

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tissues (Drenckhahn et al. 1985; Nelson and Veshnock 1986; Drenckhahn and Bennett 1987). More recently, studies using siRNA have revealed that ankyrin-G and β-2 spectrin collaborate in formation of the lateral membrane of bronchial epithelial cells (Kizhatil and Bennett 2004; Kizhatil et al. 2007b). Cells depleted of either protein maintain apical–basal polarity, but fail to form new lateral membrane following initiation of cell–cell contact and remain flattened rather than columnar. Moreover, ankyrin-G and β-2 spectrin-depleted cells are incapable of de novo membrane biogenesis during mitosis (Kizhatil et al. 2007b). Ankyrin-G requires β-2 spectrin as a partner because ankyrin-G mutants lacking β-2 spectrin-binding activity are not active in restoring the lateral membrane (Kizhatil et al. 2007b). Ankyrin-G and β-2 spectrin thus work together in bulk delivery of proteins and phospholipids to the lateral membrane.

β spectrins associated with intracellular membranes were initially believed to be distinct from those associated with the plasma membrane. Beck and colleagues reported spectrin immunoreactivity associated with the Golgi (Beck et al. 1994), which was later attributed to β-3 spectrin (Stankewich et al. 1998). However, the cross-reacting protein in Golgi was subsequently determined to be syne-1 (also termed nesprin), which has spectrin-repeats but is otherwise distantly related to β spectrins (Gough et al. 2003). β-3 spectrin is very similar to β-2 spectrin, but has a more restricted pattern of expression primarily in the nervous system and especially the cerebellum (Sakaguchi et al. 1998). β-3 spectrin thus may have roles in intracellular transport similar to β-2 spectrin but is not a specialized component of the Golgi apparatus. Recently, mutations in β-3 spectrin were identified as the cause of a form of spinocerebellar ataxia (SCA5) (Ikeda et al. 2006), indicating an important role in maintenance of certain neurons.

E-cadherin is a key cell adhesion molecule that is required to form the first lateral membrane domains in development and is a ubiquitous component of lateral membranes in epithelial tissues. E-cadherin has recently been reported to bind to ankyrin-G through a highly conserved site in its cytoplasmic domain and to require ankyrin-binding activity for efficient exit from the trans Golgi network (Kizhatil et al. 2007a). Moreover, both ankyrin-G and β-2 spectrin are required for accumulation of E-cadherin at the lateral membrane in both epithelial cells and pre-implantation embryos. E-cadherin thus works together with ankyrin-G and β-2 spectrin to coordinate membrane assembly with extracellular interactions of at sites of cell–cell contact. Coupling of E-cadherin to a versatile adaptor protein such as ankyrin-G could promote corecruitment of diverse proteins to sites of cell–cell contact. For example, ankyrin-G associates with other lateral membrane proteins including the Na/K ATPase (Nelson and Veshnock, 1987) and the RhBG ammonium transporter (Lopez et al. 2005). It will be important to determine if ankyrin-binding activity is shared by other proteins residing in the lateral membrane.

Spectrin–actin complexes are stabilized by accessory proteins such as adducin, which recruits spectrin to the fast-growing end of actin filaments (Gardner and Bennett 1987; Li et al. 1998; Kuhlman et al, 1996), and tropomodulin, which caps the slow-growing ends of actin filaments (Littlefield and Fowler 2008). Adducin and tropomodulin are both required to stabilize spectrin–actin networks on the lateral membrane of epithelial cells (Abdi and Bennett 2008; Weber et al. 2007). Depletion of either protein by siRNA results in loss of lateral membrane height. Moreover, depletion of adducin increases long-range mobility of E-cadherin on the lateral membrane (Abdi and Bennett 2008). Adducin is phosphorylated and inactivated by protein kinase C (Matsuoka et al. 1998), suggesting the possibility of signals that modulate the stability of the spectrin-actin network in the lateral membrane. Interestingly in this regard, pleiotrophin is a cytokine that promotes adducin phosphorylation and increased proliferation of epithelial cells (Pariser et al. 2005).

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CORE MECHANISMS AND EVOLUTION OF MEMBRANE DOMAINS

Having gone into details of individual membrane domains, it is worthwhile to consider common requirements and the special features of ankyrins and spectrins that satisfy these core needs. All of the domains considered in this review depend on corecruitment of functionally related but structurally distinct membrane partners. For example, axon initial segments are enriched in voltage-gated Na channels, KCNQ2/3 channels, and 186 kDa neurofascin (Fig. 2A), lateral membranes have E-cadherin and the Na/K ATPase (Fig. 2D), and ankyrin-B-based cardiomyocyte T-tubule domains contain the Na/K ATPase together with the Na/Ca exchanger (Fig. 2C). These proteins all have independently evolved ability to bind to ankyrin. A shared feature of many ankyrin-binding sites is that they are either predicted or shown to be extended peptides lacking secondary structure. For example the anion exchanger site is an 11 amino-acid loop based on the crystal structure (Stefanovic et al. 2007), the cytoplasmic domains of E-cadherin and L1 CAMS are established to be natively unstructured by biophysical methods (Huber et al. 2001; Zhang et al. 1998), and sites of Nav channels, KCNQ2/3 channels, RhBG ammonium transporter, and β-dystroglycan are predicted to be unstructured (Fig. 3A).

A possible binding site for unstructured peptides could be the ankyrin groove that runs the 240-angstrom length of the repeat stack (Michaely et al. 2002) (Fig. 3A,B). A groove of this length with variation in surface-exposed residues could potentially accommodate multiple types of partners. Interestingly, ANK repeats can bind to more than one partner at a time and thus are capable of forming homo- and hetero-complexes (Michaely and Bennett, 1995).

Natively unstructured domains of proteins are widely used in protein recognition (Dyson and Wright 2005). One advantage of such a code is that unstructured proteins can multitask and also engage endocytic machinery and other adaptor proteins depending on cellular requirements. Another advantage is that the affinity for ankyrin can vary: The Kd for ankyrin is 10 nM for the anion exchanger, 50 nM for neurofascin, and 500 nM for E-cadherin. This variable affinity allows for flexibility depending on the physiological context. Finally, intrinsically unstructured proteins represent the most rapidly evolving part of the genome (Brown et al. 2002), and have the potential to adjust readily to new physiological demands such as the rapid acquisition of myelination.

Another emerging theme as we learn more about ankyrin-based membrane domains is that they assemble through direct targeting of components along microtubules to specific sites. A similar direct targeting pathway has been proposed for assembly of gap junction subunits at adherens junctions (Shaw et al. 2007). Direct targeting is in contrast to many current models that invoke endocytosis and transcytosis as primary mechanisms for sorting. Ankyrins can bind directly to microtubules (Bennett and Davis 1981), and also can serve as adaptors for the dynactin complex, which can stabilize the fast-growing ends of microtubules, at least in skeletal muscle (Ayalon et al. 2008). Ankyrins also stabilize microtubules at the presynaptic neuromuscular junction, and also may have roles in axonal transport (Pielage et al. 2008; Koch et al. 2008). A current mystery is how newly synthesized membrane proteins are coupled to the appropriate microtubules, especially in epithelial cells and neurons where multiple types of microtubules coexist.

In addition to their roles in stabilizing proteins at the plasma membrane and in directed transport, ankyrin and spectrin may also establish specialized membrane domains. For example, ankyrin-G and β-2 spectrin are required for biogenesis of epithelial lateral membranes (Kizhatil and Bennett 2004; Kizhatil et al, 2007b). Ankyrin-G also is required to form axon initial segments, which lose all initial segment markers and develop dendritic properties in ankyrin-G-knockdown cells (Hedstrom et al. 2008). Clues to how ankyrins and β spectrins could participate in

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bulk transport of proteins and phospholipids in assembly of membrane domains come from the findings that β-2 spectrin interacts with membrane phospholipids through multiple sites (An et al. 2004; Muresan et al. 2001), and with PI lipids through its PH domain (Hyvönen et al. 1995). Moreover, spectrins also interact with microtubule-based motors, either directly (Takeda et al. 2000), or through dynactin (Muresan et al. 2001; Holleran et al. 2001; Holleran et al. 1996). The combination of ankyrin, with its diversity in protein recognition, and β spectrin, with its capacity to connect membrane compartments with microtubule-based motors, seems well suited for segregation and transport of membrane proteins and lipids to specialized domains. A central unanswered question is the identity of the initial polarity signals that define the site of delivery for ankyrin/spectrin cargo.

SUMMARY AND PERSPECTIVES

Ankyrins and spectrins were first discovered as partners in plasma membrane of erythrocytes, but now are established to be required for specialized membrane domains in many types of cells. Spectrins can both form a two-dimensional actin-based network on the plasma membrane that restricts membrane-spanning proteins, as well as participate in microtubule-dependent transport of membrane lipids and proteins. Ankyrins serve as membrane adaptors that connect spectrin to diverse membrane-spanning proteins through recognition by ANK repeats. Currently characterized ankyrin-binding sites are short 10–20 amino acid stretches that do not have a single shared motif but are intrinsically unstructured or configured as a loop. Ankyrins, in many cases operating with spectrins, are involved in coordinate assembly of a variety of ion transporters and cell adhesion molecules at axon initial segments and nodes of Ranvier in myelinated nerves, the neuromuscular junction, T-tubule microdomains in cardiomyocytes, costameres in striated muscle, and the lateral membrane domain of epithelial cells. Cardiac arrhythmias result from mutation or deficiency of ankyrin-B

or mutation of ankyrin-G-binding site of the cardiac voltage-gated sodium channel. It is likely that additional human diseases will turn out to result from defects in organization of membrane proteins. Important questions for future work include elucidation of the polarity signals that define where these domains are localized in cells as well as resolving their assembly mechanisms involving sorting and preferential transport. It is clear that future resolution of the core mechanisms for roles of ankyrins and spectrins in membrane domains will have pervasive implications for physiology as well as clinical medicine.

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