Minireview

# Ankyrins

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**Abstract This review is focused on ankyrin which is a protein linker between the integral membrane proteins and spectrin-based cytoskeleton. Structure and distribution of different ankyrin isoforms that are products of alternative-spliced genes are described. Interaction of ankyrins with various membranes is considered. Special attention is paid to ankyrin participation in signal transduction and in assembly of integral membrane proteins in specialized membrane domains.** 

*Key words:* Ankyrin; Membrane cytoskeleton; Ankyrin repeat; Signal transduction; Protein sorting

## 1. Introduction

The membrane-associated cytoskeleton is a two-dimensional network located on the cytoplasmic surface of the plasma membrane [1,2]. It is built up mainly by flexible rod-shaped molecules of spectrin (or its analogue fodrin) that are joined by ends to form five- or six-sided polygons (Fig. 1A). The corners of polygons consist of short actin filaments. Membrane cytoskeleton is crucial to maintain the shape of the cells and to restrict the lateral diffusion of integral membrane proteins. The cytoskeleton is attached to the plasma membrane through vertical interactions involving ankyrin (Fig. 1B). Ankyrin isoforms appear to provide also the linkage between the cytoskeleton and the membranes of intracellular organelles. However, ankyrin is not a simple linker: it is also involved in the signal transduction and protein sorting.

## 2. Ankyrin isoforms

Ankyrins are a family of proteins that possess binding sites for different integral membrane proteins as well as for cytoskeleton proteins. Molecular cloning has identified three distinct genes encoding ankyrins (ANK1, ANK2 and ANK3) that are expressed as tissue specific alternatively spliced isoforms. Corresponding polypeptides are designated as Ank1 (ankyrin R), Ank2 (ankyrin B), and Ank3 (ankyrin G).

Ankyrins R and B with molecular weight ($M_r$) 206 and 220 kDa, respectively, are the best characterized members of the family. These isoforms share a similar domain structure [3]. They contain three domains: N-terminal or membrane-binding, central spectrin/fodrin-binding, and C-terminal regulatory domains (Fig. 1C).

The membrane-binding domain consists for 30% of α-helix [4]. It is formed by 24 tandemly organized repeat motifs (ankyrin repeats), each of them made up of 33 amino acids [1,3]. The membrane-binding domain has a globular shape and is formed by four independently folded sub-domains of six repeats each [5].

The spectrin-binding domain contains three sub-domains [6]. The N-terminal sub-domain is enriched in proline and acidic amino acids. The central sub-domain contains mainly basic amino acids and the C-terminal sub-domain is neutral. The first two sub-domains are highly conserved between ankyrins B and R while the acidic region is variable [3].

The regulatory domain is very acidic and sensitive to proteolysis [7,8]. It modulates affinities of both spectrin- and membrane-binding domains to target proteins [9].

Ankyrins are present as a set of isoforms in a number of tissues. The major ankyrin in human erythrocytes is ankyrin R with a $M_r$ of about 206 kDa (protein band 2.1). Ankyrins with $M_r$ 186, 170, and 145 kDa are presented in a smaller amount [1]. Most of the ankyrin isoforms result from alternative splicing, however some of them (for example, 170-kDa ankyrin) may be proteolytic fragments of ankyrins with a higher $M_r$ [9].

Ankyrin R is subjected to modification by alternative splicing at sites located in the regulatory domain [3,8,10]. The 186-kDa ankyrin R (protein band 2.2) results from the deletion of 162 amino acids in the regulatory domain (Fig. 2). It has higher affinity to ankyrin-binding proteins than 206-kDa ankyrin R [3].

In addition to erythrocytes, ankyrin R is expressed in muscles [11], macrophages [12], endothelial cells [13], in granule and Purkinje cells of the cerebellum, in neurons of the spinal cord and hippocampus [14,15].

Small ankyrins R (20–26 kDa) are alternatively spliced isoforms produced by the ANK1 gene [16,17]. They are localized in the region M and Z lines of skeletal myofibrils enriched in the sarcoplasmic reticulum. These ankyrins lack both the membrane- and spectrin-binding domains and the C-terminal domain is significantly shortened (the last 82 amino acids are retained). The N-terminal part of these ankyrins contains a unique highly hydrophobic structure including 72 amino acids forming a membrane-protruding helix (Fig. 2).

Ankyrins B are presented mainly in nervous tissue. 440-kDa ankyrin B contains a 220-kDa rod-shaped domain inserted between C-terminal and spectrin-binding domains (Fig. 2). This isoform is expressed in unmyelinated axons and dendrites

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*Abbreviations:* $M_r$, molecular weight; $IP_3$, inositol 1,4,5-triphosphate; $IP_3R$, inositol 1,4,5-triphosphate receptor; RyR, ryanodine receptor

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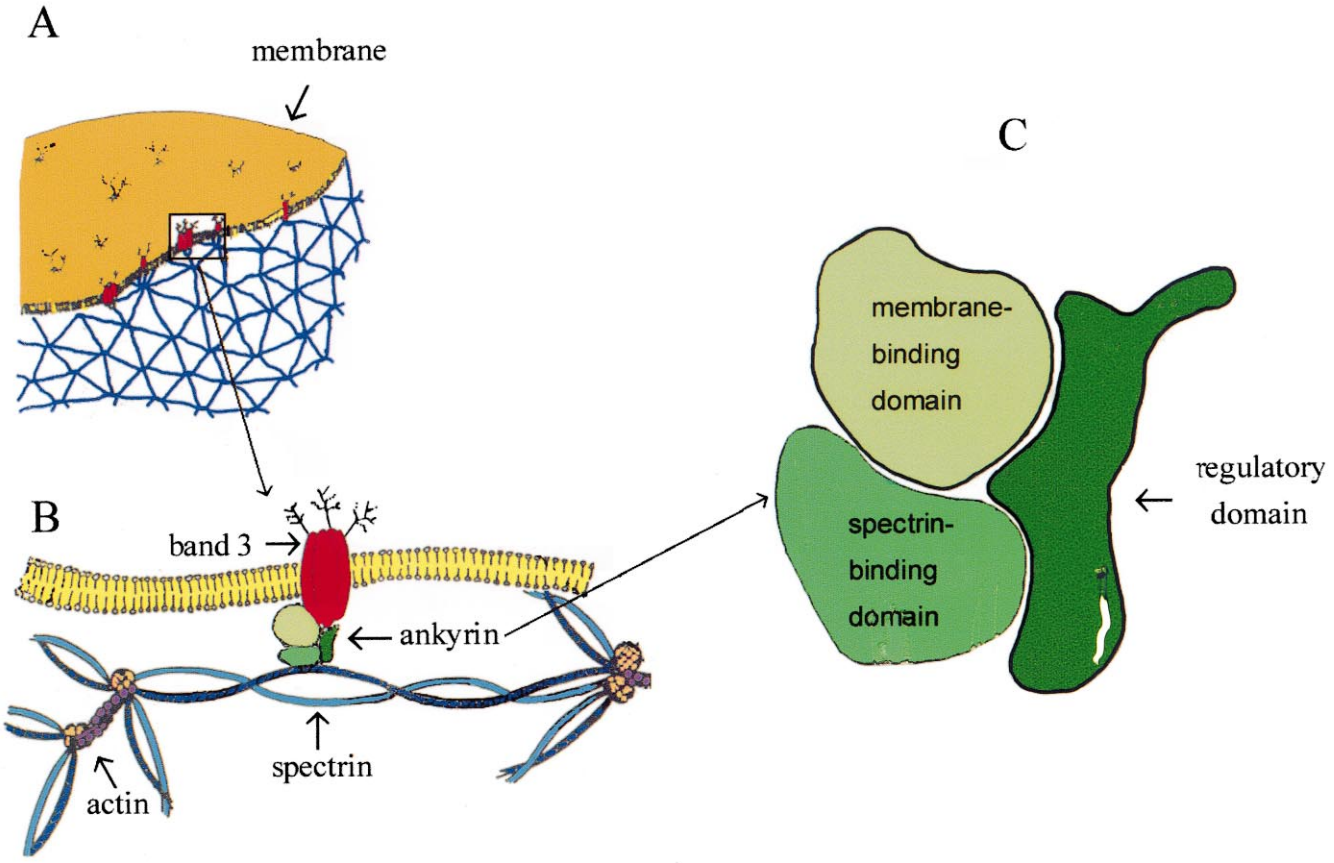


Fig. 1. Schematic models showing the organization of spectrin-based cytoskeleton (A), attachment of integral membrane protein (band 3) to a spectrin molecule mediated by ankyrin (B), and the domain structure of the ankyrin R molecule. Based on the figures presented in reviews [2] and [3].

of embryonic brain [18]. The 220-kDa ankyrin B with a domain organization similar to those of ankyrin R (Fig. 2) is expressed in cell bodies and dendrites of neurons and in glia [19].

The ANK3 gene encodes isoforms that are presented in many tissues. Ankyrins G are main ankyrin forms in epithelial cells, myocytes, hepatocytes, megakaryocytes, and neurons [20]. In brain the 190-, 270- and 480-kDa ankyrins G are found: the 190-kDa isoform is abundant in unmyelinated axons, 270- and 480-kDa isoforms are localized in the nodes of Ranvier and in the initial segments of myelinated axons [21,22].

A molecule of the 480-kDa ankyrin G consists of the globular (membrane- and spectrin-binding domains) and the extended regulatory parts (Fig. 2). A unique 46-kDa fragment enriched in serine and threonine is inserted between the spectrin-binding domain and the terminal part of the 480- and 270-kDa ankyrin G molecules (Fig. 2). This fragment is glycosylated, and glycosylation seems to be responsible for the specific localization of these isoforms [21].

Ankyrin G with a $M_r$ of about 190 kDa expressed in kidney contains a N-terminal unique fragment (31 amino acid residues), membrane-and spectrin-binding domains, and truncated regulatory domains (Fig. 2) [23]. Four isoforms of ankyrin G are expressed in kidney: 215- and 200-kDa isoforms are concentrated mainly in proximal cells, 170- and 120-kDa isoforms- in distal tubular cells of the inner medulla [24].

The 119-kDa ankyrin G expressed in epithelium and muscles associates with the Golgi apparatus, other small isoforms of ankyrin G (100–120-kDa) are linked with lysosomes [25,26] and the sarcoplasmic reticulum in the region of the Z lines [27]. The 100–120-kDa ankyrins G that are found in colon, kidney, and testis lack the membrane-binding domain and its regulatory domain is shortened to 29 amino acid residues (Fig. 2B) [12].

## 3. Interaction of ankyrin with cytoskeleton proteins

A molecule of spectrin consists of two extended α- and β-subunits that twist about each other forming a helix with length of about 200 nm (Fig. 1) [28]. One ankyrin molecule is bound to one β-subunit of the spectrin heterotetramer providing a major membrane attachment of cytoskeleton network. Spectrin interacts with the N-terminal part of the spectrin-binding domain: proteolytic fragments of ankyrin missing the first 80 amino acid residues of this domain lose spectrin-binding activity [29].

Ankyrin can bind also to the protein of intermediary filaments vimentin [30] as well as to tubulin of microtubules [31]. Thus, ankyrin seems to participate in the attachment of the intermediary filaments and microtubules to the membrane.

## 4. Interaction of ankyrin with membrane proteins

Ankyrin interacts with cytoplasmic domains of several integral membrane proteins. Mainly membrane-binding domain

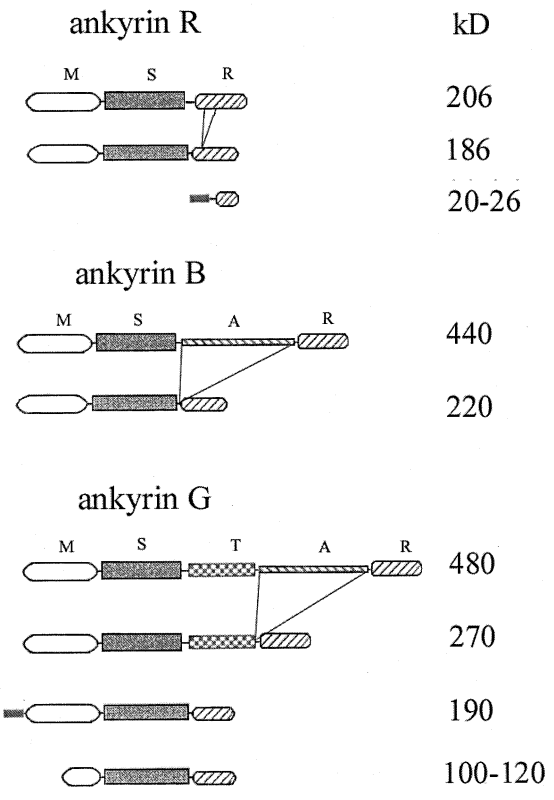


Fig. 2. Domain structure of ankyrin isoforms. M – membrane-binding domain; S – spectrin-binding domain; A – extended rod-shaped domain; T – threonine and serine enriched domain; R – regulatory domain.

of ankyrin participates in the binding. However, in some cases spectrin-binding [32] and regulatory domains are involved in the interaction with the membrane proteins, it is mainly characteristic for ankyrin G [33]. One ankyrin molecule is able to bind several various membrane proteins providing assembly of integral membrane proteins into specialized regions of the plasma membrane [34].

*4.1. Protein 3*

$HCO_3$/Cl exchanger is a major site of ankyrin binding in erythrocyte membrane. Membrane-binding domain of ankyrin R has two binding sites for protein 3 [35]. The binding of ankyrin with the first site is due mainly to electrostatic forces and with the second site mainly to non-ionic interactions. Ankyrin binding induces the oligomerization of protein 3 [36]. About 40% of all molecules of protein 3 in erythrocytes are characterized by restricted rotational mobility that appears to be the result of their association with the cytoskeleton.

Ankyrin binds to protein 3 when it is still in endoplasmic reticulum or in the first compartment of the Golgi apparatus and ankyrin seems to be responsible for the exit of this complex from the Golgi apparatus [37].

*4.2. Na-channels*

Amiloride-sensitive Na-channels are co-localized in brush border of kidney epithelium with ankyrin, fodrin, and actin. Ankyrin binds to the 150-kDa subunit of the channel. Lateral mobility of Na-channels is restricted due to the interaction with membrane cytoskeleton [38].

Voltage-dependent Na-channels are concentrated in the initial segments of neurons, nodes of Ranvier, and in postsynaptic folds of neuromuscle junctions together with ankyrin G and spectrin [39]. Subunits β1 and β2 of voltage-sensitive Na-channels that are members of the immunoglobulin superfamily interact during cell adhesion inducing recruitment of ankyrin to the region of cell contacts [40].

*4.3. Na,K-ATPase*

Polarized localization of Na,K-ATPase in a certain part of plasma membrane (basal or apical) determines the direction of ionic transport through the epithelial layer. Na,K-ATPase is concentrated in the apical membrane of choroid plexus (secretory epithelium of brain ventricles) and retinal pigment epithelial cells. In kidney and salivary glands epithelial cells it is localized in the basal part of membrane. Specific localization of Na,K-ATPase in the plasma membrane of these epithelia is due to its interaction with the cytoskeleton elements [41–44].

Ankyrin binds to Na,K-ATPase with high affinity. There are two binding sites on the ankyrin molecule: one is within the membrane-binding and another one within the spectrin-binding domain [45]. There are also two binding sites for ankyrin on cytoplasmic domain of the α-subunit of Na,K-ATPase [32]. A site possessing higher affinity is formed by the peptide located between 142 and 166 amino acid residues.

It is known that the β-subunit of Na,K-ATPase has a large extracellular glycosylated domain. The β-subunit was identified as adhesion molecule [46]. Thus, Na,K-ATPase can simultaneously interact with the cytoskeleton and participate in the cell contacts. One can suggest that ankyrin binding to Na,K-ATPase or the involvement of Na,K-ATPase β-subunit in cell adhesion contact may also affect the activity of this pump.

*4.4. Receptors*

Ankyrin with high affinity and specificity binds to the 500-kDa ryanodine receptor (RyR) of T-lymphoma cells, skeletal muscles, heart, and brain. Ankyrin-binding sites of RyR are highly conserved [47]. There is evidence that the cytoskeleton plays a pivotal role in the regulation of RyR-mediated $Ca^{2+}$ release during T-lymphocyte activation [48].

Inositol 1,4,5-triphosphate receptor ($IP_3R$) located in the membrane of calciosomes of T-lymphoma cells and brain binds ankyrin R inhibiting $IP_3$-induced $Ca^{2+}$ release [49]. $IP_3R$ and ankyrin are co-localized after forming of receptor clusters as a result of the action of different ligands. Therefore ankyrin binding with $IP_3R$ appears to play a key role in the control of $Ca^{2+}$ release mediated by $IP_3R$. The amino acid sequence of the ankyrin-binding site in $IP_3R$ shares significant homology with these sites in other ankyrin-binding proteins (in particular, in hyaluronate receptor CD44) [49].

CD44 providing the cell interaction with extracellular matrix (collagen, fibronectin) binds ankyrin after the formation of patched structures of CD44 molecules in the membrane and this binding is necessary for signal transduction mediated by CD44 [50]. The ankyrin-binding site of CD44 is presented by a peptide consisting of 15 amino acids and located within the cytoplasmic domain [51]. Phosphorylation, acylation, and GTP binding to CD44 [52] activate ankyrin binding. Interaction of CD44 with ankyrin plays an important role in cancerogenesis [51,52].

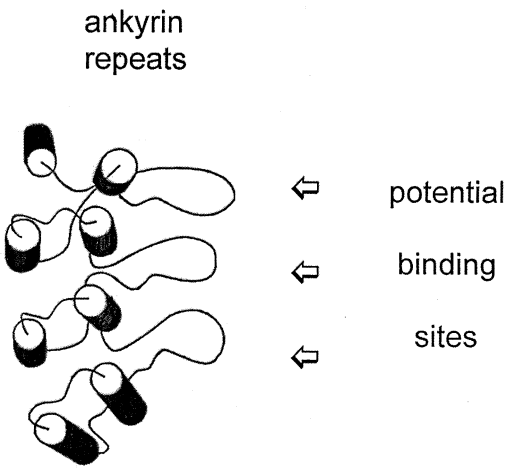


Fig. 3. Schematic model of the ankyrin repeats structure [58]. The α-helices are shown as barrels.

### 4.5. Protein kinase C (PKC)

Ankyrin, spectrin, and the β-subunit of PKC are co-localized in T-lymphocytes. PKC activation induces rapid accumulation of ankyrin, spectrin and PKC β-subunit in the cytoplasm as a single complex. T-lymphocyte treatment by PKC inhibitors results in coordinated movement of ankyrin, spectrin and PKC β-subunit to the membrane [53].

### 4.6. Cell adhesion proteins

Cell adhesion proteins of L1-family (L1, CHL1, neurofascin, Nr-CAM, and Ng-CAM) are the members of immunoglobulin superfamily. These molecules mediate cell adhesion that is required for neurite outgrowth, formation of axons, and specific growth cone guidance. The C-terminal part of the cytoplasmic domain of L1-molecules specifically binds ankyrin. The ankyrin-binding site is the most conserved region and does not share homology with any known ankyrin-binding protein [54]. Cell adhesion mediated by L1-CAM induces ankyrin recruitment in the region of cell contacts [55]. Within the ankyrin-binding site of neurofascin the tyrosine residue is located. The phosphorylation of this tyrosine prevents ankyrin binding and induces the dissociation of cell contacts [56]. Comparison of the structures of minimal ankyrin-binding sites of CD44, neurofascin, $IP_3R$ and Na, K-ATPase reveals that ankyrin binding with these proteins is due to the interaction of ankyrin repeats with the loops consisting of 5–7 hydrophilic amino acid residues [57].

## 5. Ankyrin repeats

Conserved repeats forming the membrane-binding domain of ankyrin have in general the following sequence [1,7]: -GXTPLHXAAXXGHXXXV/AXXLLXX GAXXN/DXXX-. Fifteen amino acid residues in these repeats are highly conserved and the others are variable in different repeats and ankyrin isoforms [1].

The ankyrin repeat has an L-shaped structure consisting of two α-helices following the β-hairpin loop [58]. Multiple repeats create a core inside which α-helices are concentrated. This core is stabilized by helix–helix interactions. The β-hairpins that create potential surfaces for interactions with the target proteins are exposed to the solvent (Fig. 3). The structure is stabilized by extended anti-parallel β-sheets formed between the repeats and by hydrophobic bonds inside the repeat and between the neighboring repeats. The specific feature of this structure is the array of potential binding sites created by exposed tips of β-hairpins. The sequence of this region is mostly variable.

Ankyrin repeats are identified in the hundreds of proteins that are found in prokaryotes and eukaryotes. In the list of these proteins are channels, enzymes, toxins, transcription factors [1], tankyrase, a poly(ADP-ribose) polymerase that is a negative regulator of telomere length maintenance [59], multiple proteins involved in signal transduction, in particular, integrin-linked kinases [60], inhibitors of cyclin-dependent kinases [61], death-associated protein kinase involved in apoptosis [62], and many others. This suggests that ankyrin repeats as many other conserved domains with the specific secondary structure (WD-repeats, pleckstrin homology domain, SH2- and SH3-domains) were created in the evolution as an universal module mediating the protein–protein interactions.

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