Evolution of spectrin function in cytoskeletal and membrane networks

Anthony J. Baines
Department of Biosciences and Centre for Biomedical Informatics, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

Abstract
Spectrin is a cytoskeletal protein thought to have descended from an α-actinin-like ancestor. It emerged during evolution of animals to promote integration of cells into tissues by assembling signalling and cell adhesion complexes, by enhancing the mechanical stability of membranes and by promoting assembly of specialized membrane domains. Spectrin functions as an (αβ)2 tetramer that cross-links transmembrane proteins, membrane lipids and the actin cytoskeleton, either directly or via adaptor proteins such as ankyrin and 4.1. In the present paper, I review recent findings on the origins and adaptations in this system. (i) The genome of the choanoflagellate Monosiga brevicollis encodes α-, β- and β-heavy-spectrin, indicating that spectrins evolved in the immediate unicellular precursors of animals. (ii) Ankyrin and 4.1 are not encoded in that genome, indicating that spectrin gained function during subsequent animal evolution. (iii) Protein 4.1 gained a spectrin-binding activity in the evolution of vertebrates. (iv) Interaction of chicken or mammal β-spectrin with PtdIns(4,5)P2 can be regulated by differential mRNA splicing, which can eliminate the PH (pleckstrin homology) domain in βI- or βII-spectrins; in the case of mammalian βII-spectrin, the alternative C-terminal region encodes a phosphorylation site that regulates interaction with α-spectrin. (v) In mammalian evolution, the single pre-existing α-spectrin gene was duplicated, and one of the resulting pair (αI) neo-functionalized for rapid make-and-break of tetramers. I hypothesize that the elasticity of mammalian non-nucleated erythrocytes depends on the dynamic rearrangement of spectrin dimers/tetramers under the shearing forces experienced in circulation.

Introduction
Animal cells face demands that are different from (or at least additional to) those faced by simpler unicellular eukaryotes. For example, the forces engendered by the movement of animals impose mechanical stresses on cells unlike those of simpler eukaryocytes. In animals, cells are integrated into tissues by specialized cell adhesions and by the specialization of the plasma membrane into apical and basolateral domains. Furthermore, co-ordination between cells within and between tissues requires the assembly and regulation of signalling complexes.

Among many solutions to these challenges, a system of proteins that centre on the cytoskeletal protein spectrin has proved exceedingly versatile. Spectrin and its canonical associated proteins ankyrin and 4.1 have been implicated in each of the above requirements; correspondingly, mutations in their genes or damage to the proteins lead to human disease.

For some time after their discovery in human erythrocytes, these proteins were considered to be unique to the erythrocyte [1]. However, early on, there were indications that spectrin was present in invertebrates [2]. Subsequent immunological analysis, protein purification, gene cloning and, more recently, genome analysis revealed that each of these proteins is ubiquitous in the eumetazoa (reviewed in [3]). Genetic analysis of invertebrates indicates they are essential for the normal animal life (reviewed in [3]).

Evidence that spectrin is required for resistance of animal cells to mechanical stresses comes from a variety of approaches. For example, the haemolytic anaemia hereditary spherocytosis results from deficiency of spectrin or ankyrin or their failure to assemble on the plasma membrane of erythrocytes [4]. Cells from patients with this condition are also less deformable than normal cells, and so they are removed from circulation in the spleen.

Genetic analysis of the nematode worm Caenorhabditis elegans reveals a similar requirement in muscle and nerve. Elimination of spectrin expression in this organism results in paralysis [5,6]. The structure of the muscles is greatly disrupted: the sarcomeres become disorganized, the sarcoplasmic reticulum is lost, and the muscles pull away from the body wall.

This is due to the inability of the cells to resist tension generated by myosin, since elimination of spectrin expression in a background where myosin is non-functional leads to normal muscle morphology. The nervous system is also disrupted by elimination of spectrin expression: axons break as they grow, presumably because they cannot withstand the movement of the animal, and new processes grow randomly from the nerve cell bodies to replace them [7]. Analysis of fruitflies further indicates requirement for spectrin and ankyrin in consolidation and maintenance of both pre- and post-synaptic structures [8–10].

Key words: anaemia, ankyrin, evolution, genetic disease, protein 4.1, spectrin.

Abbreviations used: ABD, actin-binding domain; AFM, atomic force microscopy; CH, calponin homology; EAAT, excitatory amino acid transporter 4; NCAM, neural cell adhesion molecule; PH, pleckstrin homology; S1H, Src homology 3.

1email a.j.baines@kent.ac.uk
Spectrin is also required for the formation of epithelia [11,12]. Knockdown of \( \beta \)-spectrin or ankyrin results in a loss of the lateral plasma membrane. The cell layer correspondingly becomes flattened. Apical spectrin (\( \alpha \) and \( \beta_{\text{heavy}} \) polypeptides) is required for fruitfly epithelial morphogenesis [13,14].

Between them, ankyrin and 4.1 bind a wide range of transmembrane proteins. In nearly all cases, this is required for their stable accumulation at the cell surface. For example, the assembly of the erythrocyte carbon dioxide metabolon requires ankyrin for the plasma membrane accumulation of its components: band 3 (an \( \text{HCO}_3^-–\text{Cl}^- \) exchanger), the rhesus proteins (putative carbon dioxide channels) and carbonic anhydrase II [15]. Similarly, 4.1 is also required for stable plasma membrane accumulation of a variety of membrane proteins including glycoporphin C and Duffy (a chemokine and \textit{Plasmodium vivax} receptor) [16].

Sodium channels in nervous system and heart interact with ankyrin and protein 4.1; this is required both for correct intracellular targeting and for normal activity. In the heart, these interactions are required for normal rhythmic heart beating [17,18]. Mechanistically, both ankyrin and 4.1 have the potential to cross-link these channels to spectrin, as well as other transmembrane proteins, including other signalling and cell adhesion molecules (see, e.g., [18–20]).

Protein 4.1 also binds to glutamate receptors, both ionotropic [21,22] and metabotropic [23], and is required for their stable cell-surface expression.

In nervous systems, interaction of sodium channels with ankyrin was an early chordate adaptation for clustering them in axon initial segments serving the generation of action potentials; at these points, ankyrin serves to co-ordinate spectrin and L1/neurofascin cell adhesion molecules, as well as the channels [19,24]. As myelin, nodes of Ranvier and saltatory conduction evolved in jawed vertebrates, potassium channels acquired an ankyrin-binding motif, allowing them to join to sodium channel clusters, whose positions were specified by neurofascin cell adhesion molecules [24–26].

In addition to the assembly of specific complexes for signalling or metabolic purposes, one possible explanation for ankyrin and 4.1 trapping so many proteins is that, by linking them to the cytoskeleton, they become relatively immobilized, thus preventing unregulated activation of signalling/metabolic pathways.

Although ankyrin and 4.1 adapt between spectrin and a very wide range of transmembrane proteins, spectrin also has a range of interactions that control membrane protein accumulation and activity. For example, the adhesive activity of the erythrocyte laminin receptor Lutheran is modulated by direct interaction with spectrin [27,28]. In the nervous system, spectrin binds, among other channels and transporters, EAAT4 (excitatory amino acid transporter 4): mutations in spectrin that abolish this interaction result in loss of EAAT4 from the cell surface and are the origin of spinocerebellar atxia type 5 [29,30]. Spectrin also modulates NCAM (neural cell adhesion molecule)-induced neurite outgrowth activity through interactions with the cytoplasmic domains of NCAM140 and NCAM180 [31].

Figure 1 shows a generalized view of complexes of spectrin, ankyrin, 4.1 and actin, with their roles in clustering membrane proteins.
In a spectrin dimer, α- and β-chains lie side-by-side and antiparallel. The bulk of the chains are made up of successive triple-helical repeats. Partial repeats at the N-terminus of α-chain and near the C-terminus of β-chain interact either to close the dimer or with another dimer to make a tetramer (or with further dimers to make higher-order oligomers). The β-spectrin N-terminal region contains a pair of CH domains that bind actin, 4.1, adducin and PtdInsP$_2$ (PIP$_2$). Triple helical repeats 14–15 bind ankyrin. Other triple helices in both chains bind a variety of ligands, including the region between repeats 9 and 11 (including the SH3 domain) which can bind both proteins and phospholipids. At the C-terminus of β-spectrin, there can be a splice variation that generates 'long' or 'short' C-terminal variants. The long variant has a PHI domain, which binds PtdInsP$_2$; this is the canonical form found throughout the animal kingdom. In some vertebrate spectrins, however, this can be removed by splicing: in the case of human βII-spectrin (indicated here), a short alternative C-terminal contains a phosphorylation site that regulates interaction with α-spectrin.

The evolutionary origins of spectrin ankyrin and 4.1

The functions of the spectrin system, such as resistance to the forces engendered by animal movement, basolateral...
membrane biogenesis and the assembly of signalling complexes, represent functions required in animals rather than their simpler eukaryotic ancestors. What is the origin of this system?

The domains that characterize spectrin (triple-helical repeats, CH domains, EF hands and SH3 domains) are all annotated in the Interpro database [53] in prokaryotes. During the evolution of eukaryotes, a process likened to “molecular Scrabble” [54] resulted in the appearance of a protein resembling α-actinin, which contained a pair of CH domains, two to four triple helices and a calmodulin-like domain [55,56]. It is generally considered that spectrin is the descendant of an α-actinin ancestor [57–59]. Successive duplications of the triple helices, followed by duplication and adaptation of chains into α, β and βHeavy variants would be a simple mechanism for evolution of a spectrin-like molecule. Similarly, other proteins that contain a pair of CH domains, triple helices and EF hands probably descended from an α-actinin ancestor to form a superfamily containing such animal proteins as dystrophin, utrophin, microtubule–actin cross-linking factor, plectin and dystonin [3].

The first appearance of spectrin seems to have been in the immediate ancestors of the animals. A group of protozoa, choanoflagellates, is widely considered to represent a group of organisms whose colonial lifestyle became adapted to form the first primitive animals (the urmetazoa) [60,61]. These organisms are characterized by a flagellum with a collar around its neck, and they can live as a free single cell or in colonies. Cells resembling choanoflagellates are found in sponges (choanocytes).

Annotation of the genome of the choanoflagellate Monosiga brevicollis [62] reveals spectrin genes. These appear to be very similar to human spectrins: the domain structures of α-, β- and βHeavy spectrin in this organism closely resemble those of human spectrins, except that the conventional β-chain is predicted to contain an SH3 domain.

Spectrin is ubiquitous in animals. All animals have a single α-spectrin gene, except mammals. Mammals have two genes, seemingly resulting from a duplication event before mammals diverged into placental, pouched and egg-laying groups. αII-Spectrin effectively represents the pre-existing α-spectrin. αII-Spectrin has diverged from αII-spectrin, and has been neofunctionalized for rapid make-and-break of tetramers. C. briggsae, Caenorhabditis briggsae; F. rubripes, Fugu rubripes; T. nigroviridis, Tetraodon nigroviridis. Figure from reprinted from [68] with permission.

**Evolution of spectrin function: structural adaptations**

The evolution of vertebrates was accompanied by two rounds of whole-genome duplication (the 2R hypothesis [67]). Thus up to four copies of genes retained from invertebrate ancestors should be found in vertebrates. Vertebrates have four conventional β-spectrins (βI–βIV). However, it was evidently not advantageous to retain all the duplicates of α- and βHeavy-spectrins. Single α- and βHeavy-spectrin characterizes the genomes of organisms such as fish, frog and chicken (reviewed in [3]).

The genomes of mammals contain two α-spectrin genes arising from a gene duplication early in mammalian evolution (Figure 3) [68]. One of these closely resembles the ancestral α-spectrin: this is known as αII-spectrin. The other, α1-spectrin, has been adapted such that, in humans, its sequence diverges from αII-spectrin more than human αII-spectrin diverges from fruitly α-spectrin.

α1- and βI-spectrin are expressed most abundantly in mammalian erythrocytes. Mammalian erythrocytes are distinct from their vertebrate precursors in that the nucleus and intracellular organelles are lost during differentiation. The enucleated cell has to survive in circulation without major biosynthetic repair mechanisms, so it might be predicted that the properties of the plasma membrane are highly adapted for resilience to the stresses of circulation. Biochemically, a major
difference between αI- and αII-spectrin is their affinity for β-spectrin in the formation of tetramers. In vitro, αI-spectrin binds βI-spectrin at the site of tetramer formation with a $K_d$ close to 800 nM [69]. This is approx. 100 times weaker than the affinity of αII- for βII-spectrin.

To test the significance of the differing biochemical properties of α-spectrins in the erythrocyte membrane, we incorporated fragments of the two different α-spectrins into erythrocyte ghosts and measured their resilience to mechanical shear [68]. The fragments of α-spectrin were from the N-terminus, and were designed to act as competitive inhibitors of tetramer formation. Either fragment was incubated with erythrocyte membranes in vitro at 37°C: each fragment rapidly became incorporated into the membranes, and bound to spectrin at the sites where tetramers form. Erythrocyte spectrin is normally >90% tetramer: increasing amounts of each fragment reduced the proportion of tetramers, and increased the proportion of dimers (with the fragment attached). Measurement of the stability of the erythrocyte membrane after incorporation of the fragments showed that the αII fragment destabilized the membranes at much lower concentrations than the αI fragment. From this we conclude that (i) erythrocyte spectrin tetramers open and close even in the absence of added mechanical shearing forces, and (ii) under shear forces similar to those found in circulation, erythrocyte tetramers dissociate rapidly. Erythrocyte tetramers are required for the stability of the erythrocyte membrane, but they seem to accommodate mechanical shear forces by allowing the membrane to deform elastically by rapidly making and breaking new tetramer structures. Thus αI-spectrin has been neofunctionalized in evolution to allow the rapid make and break of tetramers.

Erythrocyte spectrin has also been adapted through changes to the stability of its constituent αI- and βI-polypeptides. Erythrocyte spectrin melts in thermal denaturation experiments at a lower temperature than spectrin isolated from brain (which comprises mainly αI and βII polypeptides); this is reflected in the melting temperatures of its constituent repeats [70]. Strikingly, however, some of the repeats in βI-spectrin are comparatively unstable even at physiological temperature [70,71]. A recombinant construct of repeats 5–9 of βI-spectrin contains five full triple-helical repeats. AFM (atomic force microscopy) has been used to probe the folding state of these triple helices: pulling on the construct in the AFM at 23°C gives five peaks of resistance to pulling, i.e. five separate unfolding events. One of these is lost when the experiment is undertaken at 30°C, and one more at 33°C. In thermal denaturation experiments, repeats 8 and 9 unfold below 37°C, so it seems likely that these repeats within the central region of βI-spectrin are loosely folded in vivo [71]. It certainly appears that repeats 8 and 9 unfold on the erythrocyte membrane when it is subjected to shearing stress: under shear, a cysteine residue in the linker region between repeats 8 and 9 becomes available to thiol reagents [72].

It is also notable that the individual triple-helical repeats of spectrins generally show more sequence identity with corresponding repeats in other species than they do with other repeats in the same spectrin polypeptide [73,74]. This argues that individual repeats have been adapted for very particular purposes, and that this adaptation occurred in early animals. The particular binding activities of repeats 14 and 15 of β-spectrin for ankyrin were noted above, and similarly the protein- and lipid-binding activity of repeats in α-spectrin.

The spectrin triple-helical repeats represent a remarkably adaptable structural platform [75]. This platform has been adapted for use as a linker to give space between actin-binding and calmodulin-like domains, to act as a spring, and to act as binding sites for a diverse range of proteins and some phospholipids. Moreover, Hsu and Goodman [76] have reported that two cysteine residues in αI-spectrin repeat 21 are associated with a ubiquitin ligase activity, indicating that enzymatic function is also an adaptation of the triple helix.

**Evolution of spectrin function: regulatory adaptations**

β-Spectrin contains a C-terminal PH (pleckstrin homology) domain [77]. This binds PtdInsP$_2$ [78,79], and is critical for the correct targeting of spectrin in fruitfly epithelia, but probably via mechanisms distinct from PtdInsP$_2$ binding [79]. However, the first β-spectrin to be identified, that of erythrocyte, lacks the PH domain [80]. Instead there is a short C-terminal sequence rich in phosphorylation sites [81]. This arises from differential mRNA splicing of βI-spectrin: in tissues such as muscle, βI-spectrin is expressed with the PH domain [82]. The function of spectrin phosphorylation in erythrocytes is still not defined 30 years or so after it was first investigated, although it is associated with membrane mechanical properties [83]. In the case of βII-spectrin, a separate evolutionary event also resulted in the appearance of splice variants in the land-based vertebrates [84,85]. As with βI-spectrin, this eliminated the PH domain, and replaced it with a short C-terminal sequence. Within this, a threonine residue can be phosphorylated by both protein kinase A and protein kinase CK2 [85]. This phosphorylation controls the interaction of α-spectrin with β-spectrin: phosphorylation reduces the affinity of αII-spectrin for short C-terminal βII-spectrin by approx. 100-fold. Experiments incorporating recombinant fragments of short C-terminal βII-spectrin into PC12 cells indicate that this phosphorylation event occurs during the process of axon outgrowth, and that spectrin tetramers are dynamically regulated in this process.

Phosphoproteomics indicates that all the human spectrin chains are phosphorylated at numerous residues that are distinct for each gene product [86,87]. It seems likely that future research will identify distinct regulatory roles for phosphorylation in each spectrin subunit.

**Further adaptations in the spectrin-4.1-ankyrin system**

It would be surprising if the evolution of enucleated erythrocytes was accompanied only by alterations in α-spectrins. However, to date, no specific mammalian...
adaptations of the 4.1 or ankyrin genes expressed in erythrocytes have been reported.

Nonetheless, the evolution of vertebrates seems to have been accompanied by other adaptations. Protein 4.1 binds both spectrin and actin, and, correspondingly, it promotes the interaction of spectrin with actin [38,88]. This activity is contained within a spectrin–actin-binding domain [89]. Analysis of the fruitfly 4.1 protein (encoded by the coracle gene) revealed that it lacks this domain [90]. Since all four of the vertebrate 4.1 genes contain the spectrin–actin-binding domain [91], it seems that the evolutionary event that led to the appearance of this domain occurred before the two rounds of whole-genome duplications. The origins of this domain are not clear; it has a rather low amino acid complexity, and both BLAST and Interpro searches do not reveal its origin. Protein 4.1 has two further domains [FERM (4.1/ezrin/radixin/moesin) and C-terminal domains] that each bind membrane proteins [63,92], so 4.1 can act as a membrane cross-linking protein in the absence of cytoskeletal attachment.

There are no indications yet of intermediary forms of genes representing the transition between α-actinin and spectrin in simple eukaryotes. Furthermore, the function of spectrin, in the absence of ankyrin, remains to be clarified. Genetic analysis of M. brevicollis will no doubt be informative in this respect.

Perhaps one of the most exciting questions that remains to be answered is what further adaptations occurred in the evolution of enucleated mammalian erythrocytes. The small size and elasticity of mammalian erythrocytes are often considered to allow the very rapid circulation that supports processes characteristic of mammalian life, such as homoeothermy and placental development of the young. Thus understanding the adaptations that allow human erythrocytes to survive in circulation for 120 days is of great interest both for understanding our evolution, disease processes and also for technological purposes.

In collaboration with Mohandas Narla’s group at the New York Blood Center, we have initiated production of mice in which the site in αII-spectrin that binds β-spectrin is replaced with that from αII-spectrin to test the hypothesis that mammalian erythrocyte flexibility requires dynamic tetramers: this will enable us to investigate the significance of the dynamics of spectrin tetramer–dimer transition in vivo.

In relation to biotechnological applications, the shear forces that occur in stirred bioreactors can lead to the breakdown of recombining cells during the production of therapeutic proteins. There are probably lessons to be learned from the erythrocyte in the development of shear-resistant cells for production purposes.

Acknowledgements

I am grateful to Dr Mohandas Narla, Dr Xiuli An (both from New York Blood Center, New York, NY, U.S.A.), and Dr Martin Ridout and Ms Gayathri Advani (both from the Centre for Biomedical Informatics, University of Kent) for help and discussion.

Funding

Work in my laboratory is supported by the Biotechnology and Biological Sciences Research Council.

References


Received 25 January 2009
doi:10.1042/BST0370796