Spectrin: on the path from structure to function

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New structural analyses of the spectrin family of actin cross-linking proteins are providing molecular explanations for both the interchain binding between the α and β chains of spectrin and the intermolecular associations between spectrin and other proteins. Additionally, the analyses bring into focus a conformation which may explain aspects of spectrin's interaction with lipids.

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Abbreviations

LELY	low expression allele
HE	hereditary elliptocytosis
IP_3	inositol 1,4,5-triphosphate
PH	pleckstrin homology
PIP_2	phosphatidylinositol 4,5-bisphosphate

Introduction

It has been difficult to prove whether spectrin's mechanical properties, which have been extensively characterized for erythrocytes [1,2], are utilized in all cells that must sustain the multiple deformations and contractions of growth and development. Spectrin is clearly an essential protein [3,4••], but explaining its functional role(s) requires a molecular understanding of its structural features, in particular those of its repeating segments and its protein-binding and lipid-binding sites. This review will focus on structural and functional studies that offer the hope of just such explanations. Features of the spectrin molecule that are common to most classes of spectrin will be emphasized.

Organization of the spectrin molecule

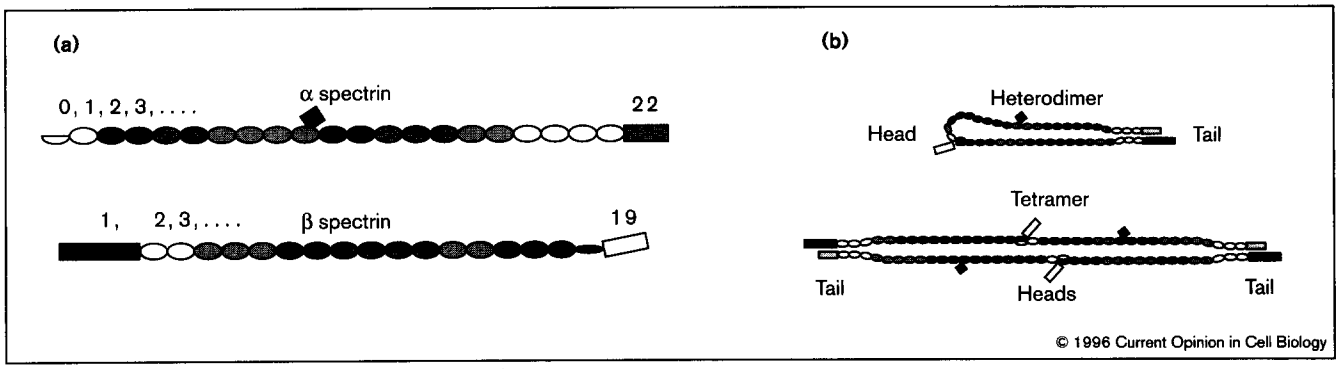
The formation and integrity of the membrane-associated supporting network that spectrin molecules can form depend on intermolecular and intramolecular associations at two key points in the spectrin molecule. At the 'head' end, interchain binding between the α and β chains (which are shown in Fig. 1a) that make up the spectrin molecule associates these chains into heterodimers or tetramers (Fig. 1b). At the 'tail' end, sites of interchain binding between spectrin chains (see Fig. 1b) integrate spectrin tetramers into a network by means of associations with protein 4.1, actin, and other proteins (see below). Between these head-end and tail-end sites, much of spectrin's length is accounted for by tandemly repeated sequence motifs, or 'segments' (Fig. 1a), each of which contains 100–120 amino acids [5]. The phasing of these tandem repeats in terms of the conformational units to which they give rise [6] has now been unambiguously established from the crystal structure of one of these repeating segments [7] (see Fig. 2). Historically, the manner of displaying and referring to the repeating segments was based on sequence alignments [5] and placed the beginning and end of each motif out of phase (by ~30 residues) with the segments that have now been defined biochemically [6] and structurally [7]. Because function depends on the relevant structural entity, we display and refer to spectrin's repeating motifs as defined by Yan *et al.*'s structural analysis [7]. To avoid the overly specialized nomenclature that is likely to evolve as the structural and functional differences between motifs are discovered, we refer to each recognized unit of spectrin as a segment, starting with segment α0 and β1 at the amino terminus of each spectrin chain (Fig. 1a).

Structure of the repeating segments

Although Yan *et al.* [7] deduced the structure of spectrin's repeating segments on the basis of only those helix contacts that were actually seen in the crystal, the expected three-helix bundle of a repeating segment was, in the crystal, formed by two interacting segments that formed a dimer containing two three-helix bundles (see Fig. 2a) Thus, to deduce the three-helix bundle structure (see Fig. 2b), Yan *et al.* used model building to insert a hairpin loop between helix B and helix C, although in the crystal, helix C was continuous with helix B and did not fold back on helices A and B (see Fig. 2a). Although modeling a loop between the B and C helices provided the only plausible structure for a segment in the native protein, the crystallography did not explain why dimers were formed with no loop between helices B and C, and the evidence could not rule out the possibility that, in solution, the same segment could contain a continuous, extended B–C helix.

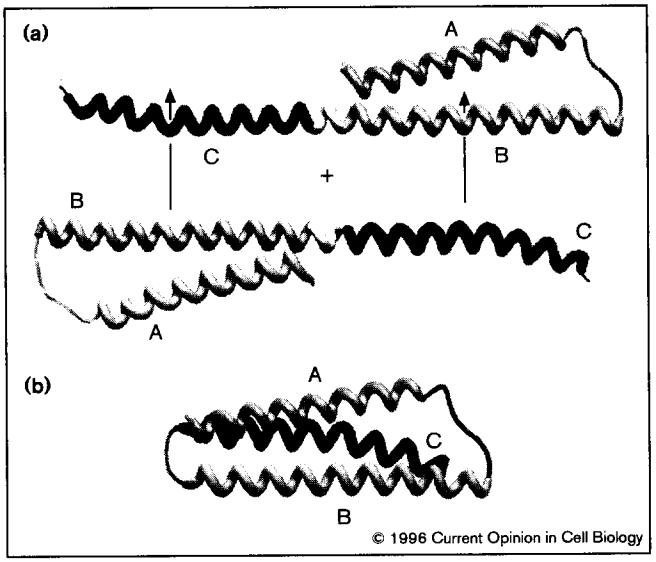
Sedimentation equilibrium analyses, in addition to non-denaturing gel electrophoresis, have now shown that, in solution, the spectrin segment used for the crystallographic analysis undergoes reversible dimerization (G Ralston, T Cronin, D Branton, unpublished data). Equilibrium between monomer and dimer populations occurs fairly rapidly above 20°C, but at a negligible rate at ~5°C. The fact that the equilibration process is temperature dependent is consistent with the requirement for extensive disruption of helix–helix packing as the reaction proceeds in either direction. The very slow equilibration at low temperatures made it possible to characterize the hydrodynamic properties of both the monomer and dimer species. A frictional ratio of 1.2 for the monomer indicated a relatively globular structure, consistent with the model

Figure 1



Spectrin architecture. **(a)** Segment nomenclature for the α-spectrin and β-spectrin chains, both of which are cartooned with their amino terminals at the left. The repeating segments are shown as ovals, the non-repeating segments as rectangles, and segment α10, a Src homology 3 (SH3) domain, as a square that protrudes from segment α9. Different shading within the ovals emphasizes that the repeating segments are similar but not identical. The ovals representing α0 and β18 are narrowed to indicate partial segments which contain only those residues that could form either one α helix (in α0) or two α helices (in β18) rather than the usual three α helices found in the complete segments. **(b)** Interchain associations between the antiparallel spectrin chains give rise to heterodimers (top) whose 'head' and 'tail' ends are defined by the manner in which they associate (in a head-to-head manner) to form tetramers (bottom).

Figure 2



Structure of the repeating segments of spectrin. **(a)** Structure of the conformational unit seen in the crystals formed by dimers of *Drosophila* spectrin segment α14. The A, B and C helices are labeled; the second polypeptide associates with the first to form a dimer containing two three-helix bundles, as indicated by the arrows. **(b)** Structure of a repeating segment monomer with one three-helix bundle, deduced from the crystal structure shown in (a).

developed by Yan *et al.* [7]. On the other hand, the frictional ratio of 1.5 for the dimer was close to that predicted for a model based on the actual crystal structure of the dimer. These results show that the crystalline dimer structure with an extended B–C helix can exist in solution, but that in the monomer or the native molecule, the C helix must be packed against the A and B helices, as shown in Figure 2b.

Head end interchain binding

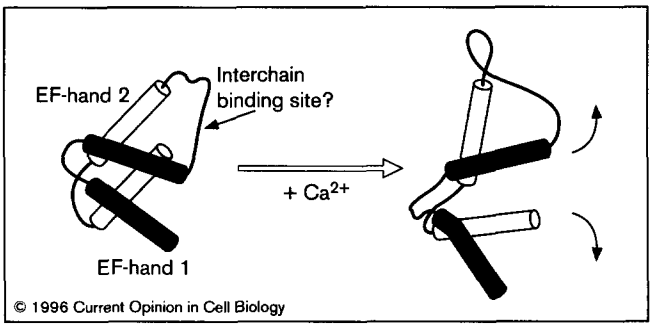
Studies of the sites within spectrin that are accessible to proteolytic degradation have been extraordinarily important in advancing our knowledge of spectrin. Indeed, while investigating a mutation in β spectrin that affected α spectrin's susceptibility to proteolysis, Tse *et al.* [8] hypothesized that the head-end association between α and β spectrin could account for α spectrin's susceptibility to proteolysis. They hypothesized that the A and B helices contained in the ~60 residues of the partial segment (β18; see Fig. 1a) found near the carboxyl terminus of β spectrin formed a three-helix bundle by packing against the potential C helix in the ~30 residues of the partial segment (α0) found at the amino terminus of α spectrin. This hypothesis has been amply verified by genetic and biochemical studies that have precisely identified the locations of the residues and motifs required for head end interchain binding.

At the genetic level it has been found that many functionally important consequences arise from deletions and mutations within the region of α spectrin that forms the C helix of a partial segment, and within the regions of β spectrin that form the A and B helices of a partial segment [9]. These consequences include effects that range from from mild to severe, both in flies (in which developmental arrest occurs) and in humans (in whom effects include hereditary elliptocytosis and non-immune hydrops fetalis) [10••,11•,12]. These effects can, in general, be attributed to a weakening or breakdown of the spectrin network as a result of head end interchain binding defects.

At the biochemical level, direct binding assays and protease footprinting assays, which use peptide fragments of native and recombinant α spectrins and β spectrins,

confirmed that the amino acids near the carboxyl terminus of β spectrin (amino acids which are homologous to those of helices A and B) and amino acids near the amino terminus of α spectrin (which are homologous to those of helix C) are the essential residues required for head end interchain association [10••,13,14,15••]. A remarkable discovery was that the binding site within β spectrin for the lone C helix near the amino terminus of α spectrin can be recreated within presumably any β spectrin repeating segment by simply deleting the segment's C helix [15••]. This discovery supports the notion that interchain binding depends on the same helix–helix interactions that produce the three-helix bundle of the other α-spectrin and β-spectrin repeats. But, although there is no doubt that the C helix contributed by α spectrin is necessary for interchain binding, it is not sufficient [14]. Other residues, found beyond the regions that are the homologues of the A, B and C helices of most repeating segments, are also required [13,16].

Figure 3



Effect of calcium binding on the amino-terminal domain of segment α22 (adapted from Trave *et al.* [19••]). Ca^{2+} binding shifts the EF-hands 1 (in gray) and 2 (in white) from a closed position to a more open one (closed arrows represent this movement), probably altering the conformation of the connecting loop (dark line), which is conserved in spectrin and α actinin but is absent in other calmodulins.

Calmodulin-like domain

The sequence of the non-repetitive segment at the carboxyl terminus of α spectrin (α22) is homologous both to segment 4 of α actinin [17] and to calmodulin [18•]. Sequence analysis predicts the presence of four EF-hands (EFs 1 to 4) within this non-repetitive, carboxy-terminal segment of α spectrin. NMR studies have shown that, in the absence of calcium, EF-1 α helices are tightly packed, whereas the EF-2 helices are less compact and are involved in side-to-side interactions with the EF-1 helices [19••,20]. Ca^{2+} binding causes a redistribution of hydrophobic interactions within EF-1, resulting in an opening of the helix–turn–helix structure that is, in turn, propagated to EF-2 (see Fig. 3). These conformational changes may modify the interface between segments α22 and β1, and may in particular modify the loop structure between EF-1 and EF-2, which plays an important role in interchain binding at the tail end of the spectrin subunits [21••]. This may explain how Ca^{2+} regulates the interaction between filamentous (F)-actin and spectrin [19••].

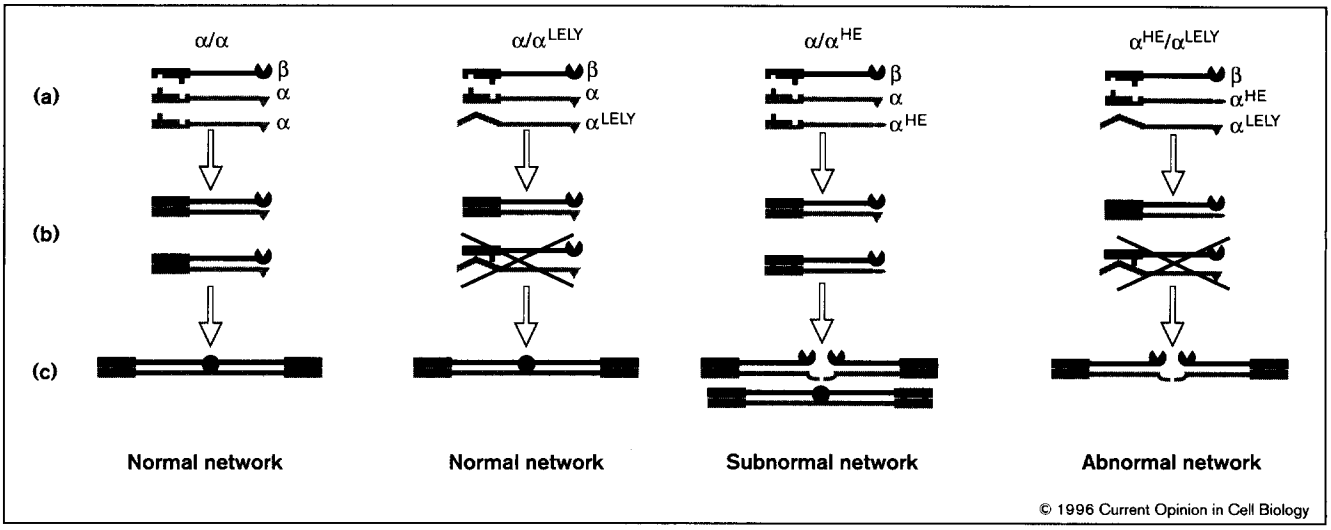
The importance of segment α22 has been further demonstrated *in vivo*: in *Drosophila*, a deletion of the carboxyl terminus of α22, which includes the deletion of EF-hands 2–4, is lethal [3]. As this deletion does not affect interchain binding [21••], other explanations for its lethal effects must be sought. Such explanations should provide new insights into how this region of spectrin can interact with, and perhaps regulate, the network it forms with protein 4.1 and actin.

Tail end interchain binding

In addition to an interaction between α22 and β1, tail end interchain binding also involves segments α20 and α21, and β2 and β3 [21••,22], which share limited sequence similarity with other segments where one segment is linked to the next by an octamer insert that is also found between α19 and α20. Deletion or duplication of conserved octamers found between α20 and α21 and between β2 and β3 results in a loss of interchain binding [21••]. Curiously, non-conservative substitutions of these conserved residues (e.g. replacement of Arg by Gly) do not affect binding [21••]. This suggests that the octamers are not themselves sites of interchain binding, but are instead critical in defining the register, or relative position, of the segments of the α-spectrin and β-spectrin chains that contain the true binding sites.

A remarkable human erythroid α spectrin variant, α^{LELY} (α low expression allele), is very common among Caucasians. It causes calamitous effects only in carriers of a mutation of the gene that is usually associated with hereditary elliptocytosis (HE), a defect that is usually attributable to alterations or deletions at the head end interchain binding sites. The α^{LELY} mutation involves a deletion of six residues at the carboxyl terminus of the predicted A helix in segment α21; this deletion should affect tail end interchain binding [23], but in humans the presence of the α^{LELY} allele by itself is asymptomatic. Surprisingly, the presence of the α^{LELY} allele reveals or potentiates head end interchain binding defects. What appears to be a remarkably long-distance effect is, in fact, explained from what is known about the formation of heterodimers and the spectrin network [24••,25] (see Fig. 4). If both the α^{LELY} and the α^{HE} mutation are in the same α spectrin chain, rejection of the α^{LELY} mutation during spectrin network formation causes the α^{HE} mutation to be rejected likewise, and hence silenced. But if the mutations occur in different α-chain alleles, rejection of the α^{LELY} mutation favors assembly of the α^{HE} mutation at the cell membrane, thus assuring a disastrously weak spectrin network.

Figure 4



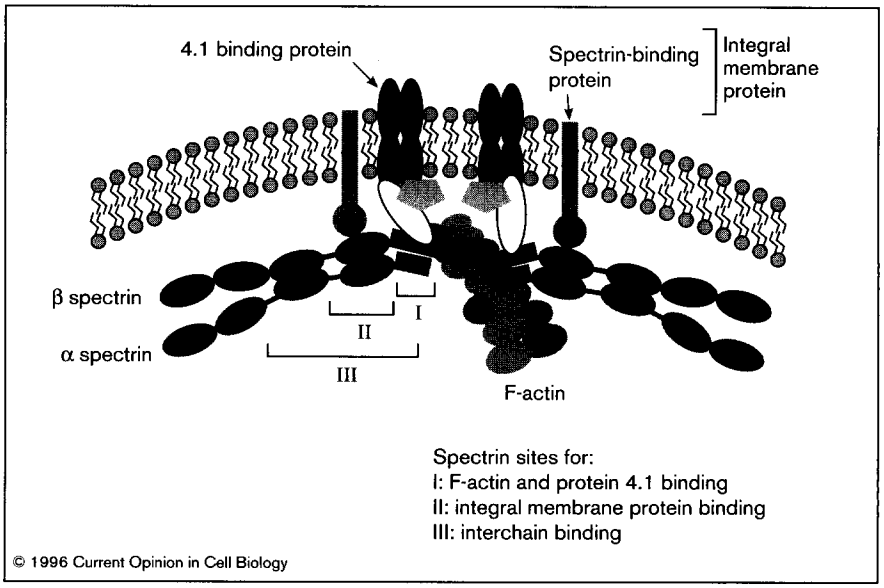
How the α^{LELY} mutation affects spectrin network stability. The columns labeled α/α and α/α^{LELY} show alleles common in most of the Caucasian population; the column labeled α/α^{HE} shows the alleles present in individuals heterozygotic for HE who may manifest some symptoms of HE; the column labeled α^{HE}/α^{LELY} shows the alleles found in individuals heterozygotic for both HE and LELY. **(a)** Chains available in the four genotypes. **(b)** Possible combinations between spectrin chains. Interactions between spectrin subunits that are initiated at the tail end of the dimer play a major role in determining which α spectrin molecules are recruited to the membrane. Recruitment of spectrins that do not contain the α^{LELY} mutation (those not crossed out) is favored. **(c)** Tetramers that form the network. If the α-spectrin chains that do not contain the α^{LELY} mutation do contain mutations at their head end interchain binding site, their preferential recruitment precludes spectrin network formation. This is because even though the genotype is heterozygous for the head end interchain binding site, recruitment disfavors α^{LELY} and thus produces an erythrocyte that is effectively 'homozygotic' for HE.

Sites of association with the cell membrane

The tail-end region of spectrin includes several binding sites that appear to overlap or be closely juxtaposed to one another (see Fig. 5). For example, the amino terminus of β spectrin associates with protein 4.1 and, through protein 4.1, with the cell membrane [26]. From the position of the Kissimmee mutation in β1 [27], a mutation which diminishes spectrin's capacity to bind protein 4.1, it appears that the 4.1-binding region is immediately adjacent to the actin-binding domain and may overlap with the interchain binding site [21••,27,28]. This overlap may explain the stabilizing action of protein 4.1 on the spectrin–actin binary complex [29].

Figure 5

Associations between the tail end of spectrin and the plasma membrane. The tail end of spectrin can be divided into three overlapping regions that are required for F-actin cross-linking and protein 4.1 binding (region I), interaction with integral membrane proteins (region II), and interchain binding (region III). Accessory binding proteins have been omitted, and the various components are not drawn to scale. As discussed in the text, other segments of β spectrin are involved in membrane association. Open ovals, protein 4.1; gray pentagon, p55; connecting bars between ovals in α and β spectrin, linking octamers.



The amino terminus of brain β spectrin also associates directly with integral membrane proteins [30••,31••], but it is not clear which residues in β spectrin form the binding site(s). According to Lombardo *et al.* [31••], the membrane-binding site of spectrin is contained in segment β2, and they suggest that the conserved five-residue motif (Gly–Lys–Pro–Pro–Lys) of β2 is a membrane-binding site. On the other hand, Davis and Bennett [30••] conclude that membrane-binding sites are located in a larger region that includes segments β3–5 and β7–8. In fact, in both studies [30••,31••], those synthetic polypeptides with the highest affinity for the membrane contained the conserved octamer between β2 and β3 that is required for interchain binding [21••]. This octamer may also constitute a membrane-binding site. If so, it may explain why non-conservative substitutions in this conserved octamer do not affect interchain binding. The conserved residues in the octamer may be required in a sequence-dependent manner as a membrane-binding motif, and in a sequence-independent manner to define the register of neighboring segments that contain the interchain-binding sites [21••]. The presence of a similar octamer between α20 and α21 implies that α-spectrin chains could also independently associate with the cell membrane. The presence of this octamer in α spectrin may explain the association of of α chains with the periphery of epithelial cells lacking β subunits [4••]. The difficulty of defining the membrane-binding site(s) in spectrin [30••,31••] may be explained by the multiplicity of spectrin target sites on the purified brain membrane used in these experiments. A more precise mapping would benefit from the identification of the target integral membrane protein(s).

It is becoming very clear that, in addition to its association with spectrin via ankyrin [32], the plasma membrane can associate with various spectrins in a variety of other ways. Associations between several integral membrane proteins (such as CD45 and NCAM180) and spectrin have been reported [33,34], and membrane lipids may attach to the pleckstrin homology domain in β19 (see below). The multiplicity of membrane-association domains on spectrin, together with the variability of spectrin ligands, may be responsible for selective targeting of spectrin isoforms to functionally distinct plasma membrane domains. Furthermore, spectrin appears to regulate the mobility of integral membrane proteins both at the plasma membrane [35•] and in intracellular membrane systems such as the Golgi [36••].

The pleckstrin homology domain

The carboxyl termini of spectrin isoforms βII and βIΣ2 contain pleckstrin homology (PH) domains, a protein module found in a large variety of proteins [37]. Although the sequence similarity between different PH domains is weak, their tertiary structure is conserved [38••,39,40]. The PH domain of spectrin participates in the anchorage of spectrin in the membrane [30••,31••]. In pleckstrin itself, phosphatidylinositol 4,5-bisphosphate (PIP_2) is a potential ligand for the PH domain and may be responsible for membrane targeting [41•]. The structure of the binding site for inositol 1,4,5-triphosphate (IP_3) in the mouse spectrin PH domain has now been resolved [42••]. IP_3 binds to the positively charged cleft between the loops connecting β strands 1 and 2, and 5 and 6. Whereas the 4-phosphate and 5-phosphate groups interact via salt bridges and hydrogen bonds and are surrounded by several positively charged residues, the inositol ring and the 1-phosphate groups are hardly involved in the interaction. On the basis of the orientation of the IP_3 molecule and the relatively weak dissociation constant for the IP_3–PH domain complex, it is probable that PIP_2, rather than IP_3, is the natural ligand for the PH domain. As the binding involves positively charged residues that are conserved in many PH domains, the interaction with PIP_2 may be a general feature of other proteins that contain PH domains. In addition to their association with lipids, PH domains may interact with proteins, as suggested by the presence of solvent-exposed hydrophobic residues on β sheets 5–7.

Conclusions

Studies of spectrin's role in the cytoskeleton have been guided by assumptions made on the basis of spectrin's function in the erythrocyte membrane skeleton, where its static mechanical properties are important [1]. But, although these mechanical properties may support and restore cell shape, spectrin's complex of intramolecular and intermolecular binding sites may also serve more dynamic, biochemical roles, especially in non-erythroid cells where spectrin's critical functions in development and human disease have only begun to emerge [3,4••,11•]. Equally penetrating insights into spectrin's multiple membrane-binding sites, and into the operation of the overlapping binding sites at the tail end of spectrin, should be forthcoming as precise maps of binding sites, together with detailed structural information, make it possible to dissect and investigate individual aspects of this large, multifunctional protein.

Acknowledgements

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Papers of particular interest, published within the annual period of review, have been highlighted as:

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- •• of outstanding interest

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Biochemical, molecular and genetic approaches are combined to map head end interchain binding sites of spectrin in *Drosophila*, and to show that a deletion of the head end binding segment, or even a single point mutation in this segment, can disrupt normal development. The results show that spectrin's capacity to form a network is a crucial aspect of its function in non-erythroid cells. The combination of biochemical and genetic approaches made it possible to examine the requirements for formation of a spectrin-based membrane skeleton during previously inaccessible stages of development.

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Binding assays using a large array of recombinant spectrin fragments are used to map, in detail, the tail end interchain binding sites of *Drosophila* spectrin. Extended regions of both the α and β chains contain multiple binding sites in both repeating and non-repeating segments. The extensive region that is involved in tail-end binding contrasts with the restricted region for head end interchain binding; the region involved in tail-end binding also clearly shows overlap of interchain-binding sites with those which bind Ca^{2+}, F-actin, protein 4.1 and the plasma membrane.

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See annotation [31••].

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In both this paper and [30••], quantitative binding assays using native and recombinant spectrin fragments were employed by the authors to discover new sites within β spectrin by which it binds to the plasma membrane. Near the amino terminus, β2 and β3 are involved, but the suggested extent to which these two segments are required for membrane association differs between the two studies. Both studies agree that the region near the carboxyl terminus which is responsible for membrane binding includes the pleckstrin homology (PH) domain in β19. These studies are the first to identify a functional role for spectrin's PH domain.

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