Introduction
A major revolution in biology has resulted in the identification and characterization of many of the components of cells at a molecular level. A significant challenge for the future is to determine how these molecules are assembled in cells and, at a higher level of organization, how the molecular ensembles of cells are able to participate in tissues. Structural proteins are likely candidates to play an important role in determining the molecular organization of cells and tissues. This paper will associate with ankyrin raise the question of how this 'evolved fit' as one mechanism for development of ankyrin-binding proteins.

Overview of brain ankyrins
Ankyrins are present in a number of tissues, but are expressed at especially high levels in vertebrate brain, where this family has been estimated to comprise from 0.5 to 1% of the total membrane protein [13, 14]. Three distinct ankyrins of brain that will be discussed here are ankyrin_b (also expressed in erythrocytes), ankyrin_{node} (the major ankyrin in brain) and ankyrin_{node}, which is found at nodes of Ranvier and axonal initial segments.

Ankyrin_{b}
This was first purified from human erythrocytes, but now is known to also be expressed in brain, with particularly high levels in the cerebellum. Ankyrin_{b} is the product of the ANK1 gene which is located on human chromosome 8p11 [15]. Ankyrin_{b} contains three domains that have been implicated in protein associations in the erythrocyte system: an 89 kDa membrane-binding domain that contains the binding site for the anion exchanger [16]; a 62 kDa domain that associates with spectrin [17]; and a regulatory domain of 55 kDa that modulates activities of both binding domains [18]. The complete sequence of human ankyrin has been deduced from analysis of cDNA isolated from a reticulocyte library and encodes a protein of 1881 amino acids from a mRNA of 7 kb [19, 20]. The protein expressed in brain is the same size as erythrocyte ankyrin, while the mRNA of brain is 9 kb, implying tissue-specific differences in non-coding regions of these transcripts [20].

A striking feature of the 89 kDa domain of ankyrin is the presence of 22 repeats containing 33 residues that occur in tandem. The repeats contain 15 highly conserved and 18 variable residues. The 33-residue periodicity is rigorously maintained with the exception of the fourth repeat which is 22 residues in length. Related 33-residue motifs are present in a number of apparently unrelated proteins of broad phylogenetic distribution (see [19] for references): (i) cytoplasmic domains of membrane proteins involved in cell differentiation including Lin12 and Glp-1 of Caenorhabditis elegans, Notch
protein of *Drosophila* and Xotch of *Xenopus*; (ii) cytosolic proteins involved in cell-cycle regulation such as SW16 and SW14 of *Saccharomyces cerevisiae* and CDC10 of *Schizosaccharomyces pombe*, where the 33-residue motif was first noted [21]; and (iii) the precursor of Nflc H, a ubiquitous transcription factor [22].

The regulatory domain of ankyrin<sub>a</sub> is subject to modification by alternative splicing of mRNA. An in-frame deletion within an exon results in removal of 163 amino acids in a lower molecular mass form of ankyrin present in human erythrocyte membranes known as protein 2.2. The alternatively spliced protein 2.2 is an activated ankyrin with an increased affinity for spectrin and increased association with the anion exchanger in erythrocyte membranes [18]. Protein 2.2 also expresses a binding site for a major class of unidentified protein sites in kidney microsomes that do not recognize the larger form of ankyrin [23]. The regulatory region thus defines specificity in binding to membrane sites as well as modulating affinities. The phenomenon of alternative splicing of ankyrin mRNA is likely to involve regions in addition to the region missing in protein 2.2. For example, a highly basic stretch of 32 residues (pI > 10) located at the C-terminus of the regulatory domain is also alternatively spliced [20].

A mutant mouse model (*nb*/*nb*) has been identified that is deficient in ankyrin<sub>a</sub> in erythrocytes, as well as brain, and has provided a clear demonstration that the same gene is expressed in these different tissues [22, 23]. Normoblastosis (*nb*) is a recessive mutation in a single gene linked to the erythrocyte ankyrin locus Ank-1 on mouse chromosome 8 [24]. *nb*/*nb* mice exhibit a 90% reduction in expression of ankyrin<sub>a</sub> in erythrocytes with a phenotype of deficiency of spectrin and a severe haemolytic anaemia [24, 25]. A surprising recent development has been the finding that the ankyrin<sub>a</sub> is expressed at less than 10% of normal values, not only in the reticulocytes, but also in the brains of *nb*/*nb* mice [22, 23]. Ankyrin<sub>a</sub> is expressed in particularly high levels of Purkinje cells of the cerebellum, and is also missing from these cells in *nb*/*nb* mice over a period of months [22]. Loss of Purkinje cells is associated with progressive ataxia and other signs of cerebellar dysfunction. The use of the *nb*/*nb* mice has thus allowed the unambiguous identification of an ankyrin isoform as ankyrin<sub>a</sub> and the distinction between ankyrin<sub>a</sub> and other ankyrin isoforms in the nervous system.

Ankyrin<sub>a</sub> is highly polarized in its cellular distribution and is confined to the plasma membrane in cell bodies and dendrites of neurons, but is missing from myelinated axons [10, 23]. Ankyrin<sub>a</sub> is also highly restricted in its expression and is limited to certain neurons. Ankyrin<sub>a</sub> is present in most motor neurons in spinal cord and both major types of neurons in the cerebellum, the Purkinje and granule cells. However, in the cerebral neocortex and the hippocampus, ankyrin<sub>a</sub> is present in a minor subset of neuron cells.

An interesting question currently under investigation is the role of alternatively spliced forms of ankyrin<sub>a</sub> in brain. Preliminary observations indicate that alternatively spliced forms of ankyrin<sub>a</sub> missing the same region as protein 2.2 of erythrocytes are expressed in brain (S. Lambert, unpublished work). An experimental challenge is to develop antibodies that selectively recognize the spliced form in order to evaluate the possibilities of differential targeting of the spliced variants.

**Ankyrin<sub>b</sub>**

This is the major isoform of ankyrin in brain tissue and shares functional activities and a similar domain structure with erythrocyte ankyrin [14]. This form of ankyrin is associated with membranes and has been localized by immunofluorescence to the plasma membranes of neuronal cell bodies as well as to glial cells [10]. The amino acid sequence of the major 220 kDa ankyrin of adult human brain.
(a product of the ANK2 gene) has recently been deduced from analysis of cDNAs [26]. This information, together with the primary sequence of ankyrin<sub>n</sub>, defines consensus features of the ankyrin family. These proteins both contain highly conserved regions that include a series of 22 consecutive repeats of 33 residues in the membrane-binding domains, and the C-terminal portion of the spectrin-binding domains. The regulatory domains of the two ankyrins are quite different, consistent with the observations of distinct membrane-attachment sites for these proteins [23].

The ankyrin<sub>n</sub> gene also is subject to alternative splicing of mRNA. A novel 440 kDa alternatively spliced variant of ankyrin<sub>n</sub> has been identified that includes the spectrin and membrane-binding domains, as well as the predicted regulatory domain of the major 220 kDa isoform [26, 27]. The 440 kDa ankyrin<sub>n</sub> contains, in addition, a sequence of a predicted size of 220 kDa which is inserted between the regulatory domain and the spectrin/membrane-binding domains (Fig. 2). The 440 kDa ankyrin<sub>n</sub> has properties expected of a peripherally associated membrane-skeletal protein: it is exclusively present in the particulate fraction of brain homogenates, is extracted with NaOH and remains associated with Triton-X-100-resistant structures [27]. The 440 kDa ankyrin<sub>n</sub> has properties expected of a peripherally associated membrane-skeletal protein: it is exclusively present in the particulate fraction of brain homogenates, is extracted with NaOH and remains associated with Triton-X-100-resistant structures [27]. The 440 kDa ankyrin<sub>n</sub> is the first form of ankyrin that can be detected in developing rat brain, and begins to appear at birth [27]. Expression of the 440 kDa ankyrin<sub>n</sub> is maximal in the neonatal period of rat development, with a peak at day 10, preceding myelin basic protein and synaptophysin. The 440 kDa ankyrin<sub>n</sub> persists in adults, although the pattern of its expression is less general than in neonatal brain tissue. Adult cerebellum contains nearly the same amount as neonatal cerebellum, while very low expression of 440 kDa ankyrin<sub>n</sub> occurs in the adult brain stem.

The 440 kDa ankyrin<sub>n</sub> was localized by immunofluorescence in molecular layers of cerebellum, cerebral cortex and hippocampus, which are regions comprised mainly of non-myelinated axons and dendrites with relatively few glial cells or neuronal cell bodies [27]. Conclusions inferred from the morphology are that the 440 kDa ankyrin<sub>n</sub> is expressed by neurons and is targeted to axons and possibly dendrites. The 440 kDa ankyrin<sub>n</sub> is highly polarized in its distribution, since it is absent from cell bodies of cerebellar neurons known to be major contributors of axons and dendrites to the molecular layer. Other brain ankyrin isoforms are present in these neuronal cell bodies, suggesting that confinement to neuronal processes is a special feature of the 440 kDa isoform.

The presence of the 440 kDa ankyrin<sub>n</sub> in non-myelinated axons, and possibly dendrites, and its high expression during a relatively short period of development, suggest that this isoform performs a specialized role in brain. Possibilities include functions related to the particular requirements of non-myelinated axons, such as the physical support of their plasma membranes; the stabilization of contacts with adjacent axons in the bundles that can be visualized by electron microscopy, and in the formation of multiple synapses with dendrites. The sequence that is unique to the 440 kDa isoform presumably is required either for preferential targeting to axons and/or for a specialized role in these structures. It will be important in future work to elucidate the complete sequence of 440 kDa ankyrin and evaluate the functional properties of the additional regions.

**Ankyrin<sub>node</sub>**

A third form of ankyrin, closely related to ankyrin<sub>n</sub>, as judged by immunological criteria, is localized on the cytoplasmic surface of axonal plasma membranes at nodes of Ranvier [10]. Ankyrin<sub>n</sub> subtypes in brain have been distinguished using mutant normoblastic mouse (nb/nb) with very low expression of ankyrin<sub>n</sub> and with antibodies selective for a single exon of ankyrin<sub>n</sub> [23]. Ankyrin<sub>n</sub> as noted above, is missing in normoblastic (nb/nb) mice. However, another isoform of ankyrin<sub>n</sub> is present in axonal initial segments and the nodes of Ranvier in myelinated axons. The isoform of nodes of Ranvier and axon initial segments is retained in nb/nb mice and does not cross-react with antibody selective for ankyrin<sub>n</sub>. Ankyrin at the nodes of Ranvier and initial segments thus may represent product(s) of a distinct gene, although the possibility that it is the product of an alternatively spliced ankyrin<sub>n</sub> pre-mRNA not altered by the nb mutation, cannot be excluded. In either case, ankyrin at the node of...
Ranvier and axon initial segments is a distinct polypeptide from ankyrin in neuron cell bodies, and represents the first example of a cytoplasmic protein that is uniquely targeted to these specialized domains. The voltage-dependent Na⁺ channel associates with ankyrin and is concentrated in the same membrane domains. These considerations suggest that the nodal form of ankyrin is a candidate to participate in either targeting or maintaining the Na⁺ channel at nodes of Ranvier and axon initial segments.

Mapping the binding sites of ankyrin

Two questions have been addressed regarding active sites of ankyrin involved in association with integral membrane proteins: (i) does ankyrin contain common or distinct sites for these proteins? and (ii) do the 33-residue repeats noted as a common feature of the ankyrin family have a role in binding? Examination of the binding sites of ankyrin for the anion exchanger and Na⁺/K⁺-ATPase supports the view that ankyrin-binding activity evolved independently at least in the case of these proteins [30]. The anion exchanger binds exclusively to the 89 kDa domain [16], while the Na⁺/K⁺-ATPase binds only weakly to the 89 kDa domain and also associates with the spectrin-binding domain. The Na⁺/K⁺-ATPase thus may require contacts with two and perhaps more domains of ankyrin to form the high Kᵢₐ complex observed with intact ankyrin.

The anion-exchanger-binding site of ankyrin within the 89 kDa domain of human erythrocyte ankyrin is retained by proteolytic fragments containing only 33-residue repeats [16]. The 33-residue repeats thus play a major role in the association of ankyrin with the anion exchanger. The issue of whether the repeats are equivalent with respect to binding to the anion exchanger has been explored using defined regions of human erythrocyte and brain ankyrins expressed in bacteria [31]. The conclusion was that the repeats are not interchangeable and that repeats 21 and 22 are essential for high-affinity binding between erythrocyte ankyrin and the anion exchanger.

The finding that specificity in association between ankyrin and the anion exchanger is provided by a rather small region of sequence raises the question of what is the function(s) of the remaining 20 33-residue repeats of the 89 kDa domain. One possibility is that the other 33-amino acid repeats interact with proteins distinct from the anion exchanger. The ability to interact with multiple proteins is not required in the context of the nuclear mammalian erythrocyte. However, the complex environment of the nervous system could provide a variety of potential ankyrin-binding proteins.

Tubulin is a candidate for an additional protein that interacts with ankyrin 33-residue repeats. Ankyrin has been previously noted to associate with microtubules and with tubulin dimers [14]. Tubulin has recently been demonstrated to associate with the repeat regions of erythrocyte ankyrin, including repeats that do not bind to the anion exchanger [31]. Association with tubulin was approximately the same for the 89 kDa domain and the polypeptide fragments containing 12 repeats, but was reduced to 10–15% in a polypeptide fragment with 6 repeats. Although further studies will be required to determine if tubulin associates preferentially with certain repeat sequences in ankyrin, the available data does suggest that tubulin interacts with broader specificity than the anion exchanger and that the number of repeats is an important parameter. The physiological significance of tubulin–ankyrin interactions remains to be explored. However, a membrane-associated protein capable of interacting with and perhaps stabilizing the ends of microtubules could have a major role in determining the organization of microtubules in cells.

The 33-residue repeat motifs similar to those of ankyrin are present in a variety of proteins and these motifs presumably have some common activity. Clearly, association with proteins related to the cytoplasmic domain of the anion exchanger is not likely to be a general feature of these 33-residue motifs. Preliminary experiments also suggest that binding to tubulin may not be a feature shared by the Notch repeats, based on binding assays with the six repeats of Notch expressed in bacteria (unpublished work). It would be of obvious interest to identify a common set of molecules that interact with 33-residue repeats. Another possibility is that 33-residue repeats have features that permit association with other 33-residue repeats. Ankyrin, in this case, may have evolved 22 repeats organized in a spherical configuration [16], in order to internally satisfy repeat–repeat interactions.

Information regarding active sites of ankyrin for the anion exchanger and Na⁺/K⁺-ATPase provides some insight into possible pathways for evolution of this interacting system of proteins. One scenario consistent with available information is that the anion exchanger and Na⁺/K⁺-ATPase independently evolved to interact with ankyrin, which remained relatively unchanged. According to this view, the integral membrane proteins would be
The interaction of ankyrin with at least some membrane proteins thus could represent a secondary activity, at least in terms of evolution. A selective advantage that would promote linkage of ion channels to ankyrin would be likely to have evolved after development of ion channel activity. Another implication of an ‘evolved-fit’ mechanism is that ion channels encoded by multigene families could differ in their affinities for ankyrin and occur in different membrane domains.

**Conclusion**

Recent work has revealed considerable diversity among ankyrins in brain owing to multiple genes and alternative splicing of mRNA. Distinct binding sites of ankyrin for the anion exchanger and the Na\(^+\)/K\(^+\)-ATPase suggest that these membrane proteins independently developed ankyrin-binding activity through an ‘evolved fit’ and that ankyrin may have played a passive role in this process. The combination of multiple forms of ankyrin and the example of at least two independent evolutionary pathways of ankyrin-binding activity are consistent with a role for ankyrins in organization of diverse integral membrane proteins in a variety of specialized membrane domains. Potential clinical importance of ankyrins is suggested by the *nb/nb* mouse which is missing ankyrin\(_B\) in brain and erythrocytes and has both a severe anaemia and degeneration of Purkinje cells in the cerebellum.

Brenda Sampson is gratefully acknowledged for help in preparation of the manuscript.

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Red cell membrane cytoskeleton and the control of membrane properties
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Introduction
The material properties of the red cell membrane, in particular its unique stability towards shearing forces and its elasticity with respect to linear deformation, result from the interaction of the phospholipid bilayer with the membrane cytoskeleton that covers its cytoplasmic surface. When this protein network is extracted or destroyed by proteolysis the membrane spontaneously vesiculates. The pathways through which the proteins exert their effects on the membrane properties are not known.

Three types of interaction between the membrane and the membrane cytoskeleton can be identified, namely bridging of spectrin to band 3, the major transmembrane protein, by ankyrin [1], binding of protein 4.1 to one or more integral membrane proteins (see below), and a weak, but globally important, direct association between cytoskeletal protein and phospholipid, almost certainly phosphatidylserine [2, 3]. We consider here only the protein–protein interactions and the manner in which they might regulate the membrane properties.

Interactions between protein 4.1 and the membrane
It is well established that protein 4.1, in addition to its association with spectrin and actin in the protein network, binds strongly to the membrane [4–6]. There has been no agreement on the identity of the binding sites, which have been variously reported to be glycophorin A (GPA) [4, 6], glycophorin C (GPC), also termed glycoconnectin [7], band 3 [5] and even phosphatidylserine [8, 9]. The cell contains about $2 \times 10^5$ molecules of protein 4.1, $10^6$ molecules of GPA, $5 \times 10^6$ molecules of GPC, $1.2 \times 10^6$ molecules of band 3 and a vast molar excess of phosphatidylserine. Binding of protein 4.1 to membrane vesicles, freed of peripheral proteins, shows saturation at a concentration similar to the original content. This renders band 3 and lipid improbable as the protein 4.1 receptors. There is compelling evidence against GPA as the primary site; for example, from the wholly normal nature of red cells from which this protein is missing [9]. There are, on the other hand, abundant grounds for believing GPC to be a binding site [7, 10, 11], despite the apparently unsatisfactory numerology.

Our rebinding isotherms for protein 4.1 to 4.1-depleted inside-out vesicles show saturation at a level corresponding to the concentration of protein 4.1 originally present in the cell. Extensive proteolysis of the vesicles with trypsin, chymotrypsin, papain, pronase, Staphylococcus griseus protease or mixtures of these does not eliminate all the binding, but instead leaves a sizeable proportion (30–50%) of the sites intact. It thus seems clear that the membrane contains two types of site, only one of which is subject to proteolysis, and, indeed, intact GPC (as well as GPA) was shown by immunoblotting to have vanished from the membranes after treatment with proteases. (We make no distinction here between GPC and the minor species GPD, which has an identical cytoplasmic sequence [12].) A further means of resolving two populations of sites is to phosphorylate protein 4.1 with protein kinase C. This can be performed in situ by treating the cells with a phorbol ester, and Danilov et al. [13] have shown that it reduces the number of sites on the membrane to which the protein will bind. We have confirmed this result: protein 4.1, phosphorylated in this manner, will compete with unphosphorylated protein 4.1 only at the protease-resistant site. Proteolysed membrane vesicles, moreover, show indistinguishable binding profiles for phosphorylated and untreated protein 4.1. Thus it is a proteolytically accessible cytoplasmic element of the receptor protein(s) that contains the site, affected by phosphorylation of protein 4.1.

Abbreviation used: GP, glycophorin.