SPECTRIN AND RELATED MOLECULES

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DEDICATION

This review is dedicated to Benjamin Dwight Goodman

I. INTRODUCTION

Erythrocyte spectrin is the major component of a skeletal protein meshwork which lines the cytoplasmic surface of the red cell membrane. This structure has been termed the membrane skeleton in order to distinguish it from cytoskeletal structures which traverse the cytoplasm of nucleated cells. The spectrin membrane skeleton plays several roles essential to normal red cell physiology including maintaining the shape of the erythrocyte, allowing it the property of reversible deformability when faced with a range of dynamic shear stresses in the circulation, giving the membrane its structural stability, regulating the lateral mobility of integral membrane proteins, and controlling the transbilayer movement of phospholipids. These functions of the membrane skeleton have received extensive reviews[1-6] and therefore are not discussed in detail here. Defects in the protein components of the membrane skeleton cause red cells to take on abnormal shapes, create unstable membrane material properties, and lead to cells with reduced cellular deformability in the circulation. Detailed discussions of the biochemical defects leading to the hereditary hemolytic anemias termed hereditary spherocytosis (HS), hereditary ellipto-

cytosis (HE), and hereditary pyropoikilocytosis (HPP) can be found in numerous reviews[4,5,7,8] and are only mentioned briefly in this review.

Prior to 1981, it was commonly believed that spectrin was present only in erythrocytes. Furthermore, it was believed that the membrane skeleton was endemic only to erythrocytes, while other cells contained a cytoskeleton composed of transcellular microtubules, microfilaments, and intermediate filaments. These ideas, however, did not explain how membrane surfaces in cells other than erythrocytes maintained their structural stability, how lateral and transbilayer movements of proteins and lipids, respectively, were controlled in nonerythroid cells, or how the shape of nonerythroid cells was maintained. The dogma described here was brought under serious scrutiny when Goodman and colleagues demonstrated that molecules which were structurally and immunologically related to spectrin could be found in diverse nonerythroid cell types.[9,10] We now know through the work of many laboratories that red blood cell (RBC) spectrin is only one member of a family of spectrins which can be found in virtually every eukaryotic cell type. The best studied nonerythroid spectrin molecules are those found in brain. We have learned a significant amount over the past 5 years concerning the structure and location of neural cell spectrins, and we are getting our first glimpses of the probable functions of these molecules. We are entering a Renaissance period in the nonerythroid spectrin field in which a descriptive literature is giving way to an understanding of function. The discovery of nonerythroid spectrin has been previously reviewed,[4,11-13] and reviews on the structure and location of brain spectrin have appeared.[13-15]

In this review, we cover the composition, structure, assembly, and function of the erythrocyte membrane skeleton; the discovery of nonerythroid spectrin; the structure, location, and protein interactions of brain spectrin and chicken intestinal brush border spectrin (TW 260/240); the discovery of two brain spectrin isoforms; and the potential functions of these spectrin isoforms, including a new hypothesis concerning the role of brain spectrin (240/235) in the early events of synaptic transmission. Who could have anticipated 5 years ago that spectrinologists would be concerned with synaptic transmission? This is an intriguing time in this field, and we hope to convey the excitement in this review.

II. THE ERYTHROCYTE MEMBRANE SKELETON

A. Definition, Composition, and Ultrastructure

The major peripheral membrane proteins of the erythrocyte membrane are all found associated with the cytoplasmic membrane surface and include enzymes such as glyceraldehyde-3-phosphate dehydrogenase (band 6); skeletal proteins such as spectrin, actin, proteins 4.1a and 4.1b, and protein 4.9; and a protein of unknown function (band 4.2). The major integral membrane proteins are band 3 and glycophorin A (or α), both of which have a polar glycosylated external domain, a hydrophobic membrane-spanning domain, and a cytoplasmic polar domain. The numerical nomenclature for erythrocyte membrane proteins is based upon their mobility in SDS polyacrylamide gels. Table 1 lists the membrane proteins, along with information on their molecular weight and number of copies per erythrocyte membrane.

When erythrocyte ghosts,[18] or washed RBCs,[1,19] are extracted with nonionic detergents such as Triton X-100, greater than 50% of the membrane phospholipids and proteins are released leaving behind a filamentous reticulum with adherent lipoid sheets and vesicles. This filamentous reticulum of proteins has been termed the "Triton shell" and serves as the operational definition of the erythrocyte membrane skeleton. The protein composition of the membrane skeleton or Triton shell depends upon the extraction conditions (ionic strength, Triton X-100, temperature). When the extraction is performed at high ionic strength, a Triton shell remains which maintains the shape of the original erythrocyte, but which contains a very simple protein composition. It contains spectrin, actin, and proteins 4.1 and 4.9, which are the essential components of the two-dimensional meshwork covering the cytoplasmic surface of the

Table 1
MAJOR HUMAN ERYTHROCYTE PROTEINS

Component	Identification	Mol wt	Approx. copies/cell	Ref.
	Spectrin			
1	α Subunit	240,000	200,000	
2	β Subunit	220,000	200,000	
2.1	Ankyrin	200,000	100,000	
2.2		190,000		
2.3		180,000		
2.6		150,000		
3	Anion transport protein	95,000	1,000,000	
4.1a		80,000	100,000	16
4.1b		78,000	100,000	16
4.2		72,000	200,000	
4.9		48,000	100,000	
5	Actin	43,000	500,000	
6	Glyceraldehyde-3-phosphate dehydrogenase	35,000	500,000	
7		29,000	400,000	
7a		29,000	60,000	12
	Tropomyosin			
7b		27,000	60,000	
8		24,000	200,000	
GPα	Glycophorin α	43,000[a]	600,000	17
GPβ	Glycophorin β	39,000[a]	50,000	17
GPγ	Glycophorin γ	25,000[a]	80,000	17

[a] Molecular weights of glycoproteins have been calculated by migration of the monomer on SDS-PAGE and therefore include glycosylation and inherent errors in determination of molecular weight of glycoproteins by SDS-PAGE.

erythrocyte membrane.[20,21] At lower ionic strength, residual lipid and several accessory proteins including ankyrin, a portion of band 3, band 4.2, and glycophorin β remain with the membrane skeleton.[20,21] As is discussed later, the membrane skeleton is composed of three types of proteins: (1) skeletal proteins which form the fibrous meshwork (spectrin, actin, proteins 4.1 and 4.9), (2) linking proteins which attach the fibrous meshwork to the bilayer (ankyrin and protein 4.1 which serves dual functions), and (3) integral transmembrane proteins which traverse the membrane and serve as the attachment sites for the linking proteins (a portion of the band 3 molecules and possibly glycophorins β or α).

Recent negatively stained images of the Triton shells (Figure 1) indicate that short actin protofilaments (arrow), which are 33 to 37 nm in length corresponding to 12 to 13 actin monomers, are linked together by the spectrin tetramers which range in length from a 50-nm condensed form to a 200-nm fully extended form depending on extraction conditions.[21,22] The propensity of spectrin to condense by intramolecular self-association and then expand to a more flexible coil may be the molecular basis of the elastic properties of the skeleton and membrane which have a maximum elastic extension ratio of 3 to 4.[21,22] These electron micrographs confirm the widely accepted model of the erythrocyte membrane skeleton which has been based upon the association of purified components *in vitro*. In the subsequent section, we discuss the structure and associations of the individual membrane skeletal components.

B. Structure and Function of Components

1. Skeletal Proteins

a. Spectrin

Spectrin, initially described as the major component of low ionic strength extracts of human erythrocyte membranes, is composed of two high molecular weight polypeptides[23] which have

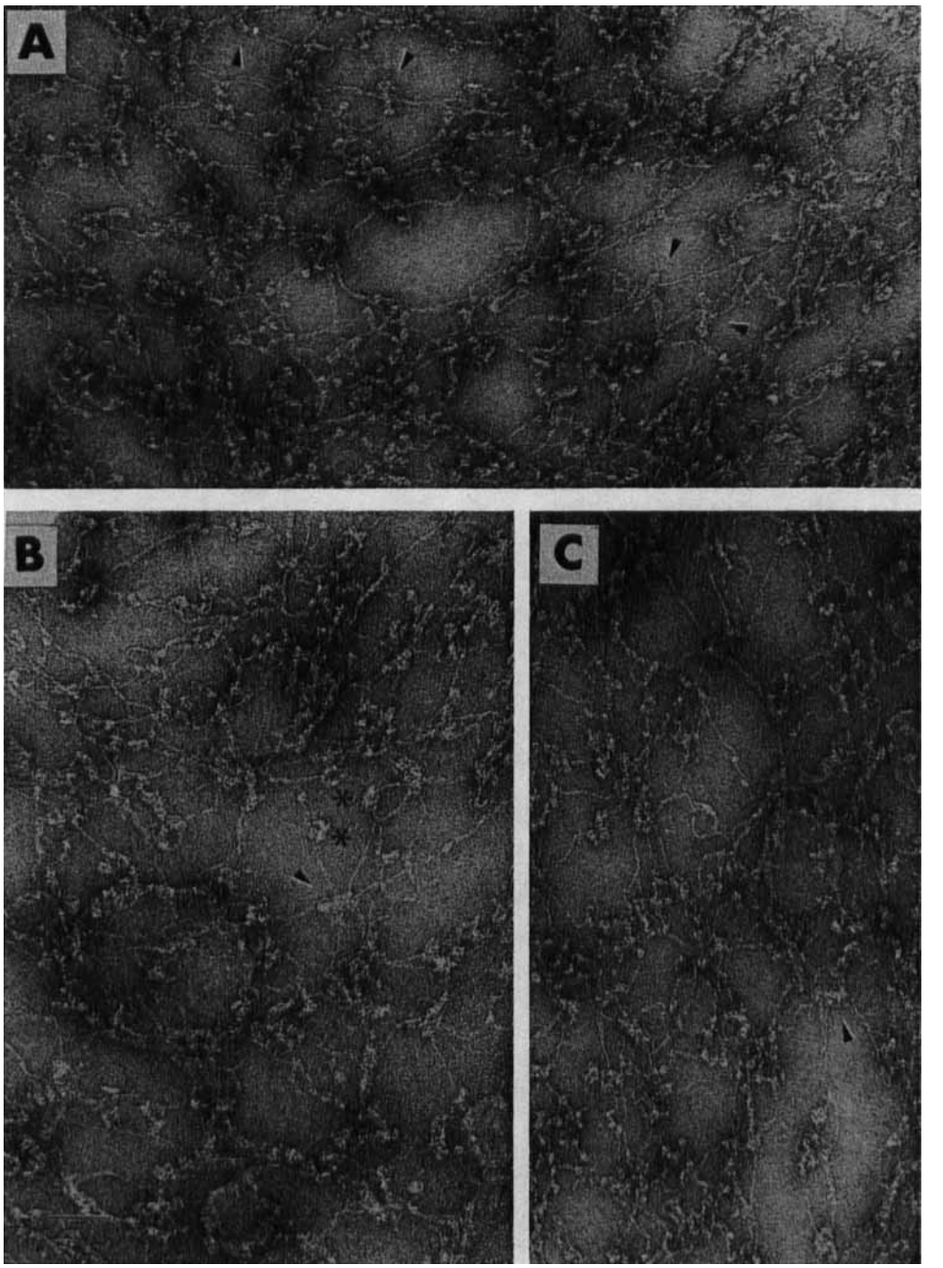


FIGURE 1. Structure of the human erythrocyte spectrin membrane skeleton. Unstripped Triton X-100 extracted membrane skeletons were prepared and expanded for visualization by negative staining and electron microscopy as described.[21] Segments of thin filaments (presumably spectrin) can be seen extending for up to 100 nm between the dense conglomerates (actin protofilaments), which are designated with arrowheads. Asterisks indicate globular complexes (presumably ankyrin-band 3) associated with the spectrin filaments. (Reproduced from Shen, B. W., Josephs, R., and Steck, T. L., *J. Cell Biol.*, 102, 987, 1986. By copyright permission of the Rockefeller University Press.)

been referred to as band 1, or the α subunit, and band 2, or the β subunit.[24,25] For mammalian erythrocyte spectrin, the α subunit has an approximate molecular weight of 240 kDa and the β subunit has an approximate molecular weight of 220 kDa, based on electrophoretic mobility. Precise molecular weights must await completion of the sequence analysis of the α and β subunits. When low ionic strength extraction of erythrocyte membranes is performed at 37°C, spectrin is eluted as an αβ heterodimer which, when isolated by gel filtration chromotography or rate zonal sedimentation, has a molecular weight of ~460,000 and a sedimentation coefficient corrected to 20°C in water ($S_{20,w}$) of 8 to 9 S. When isolated at 4°C, spectrin is primarily in the form of an $(\alpha\beta)_2$ tetramer with a molecular weight of 920,000 and $S_{20,w}$ equal to 11 to 12 S.[26,27] Spectrin dimers, tetramers, and higher oligomers are interconverted at temperatures above 29°C, but the interconversion is blocked at 4°C.[26] Therefore the extraction of spectrin in the tetrameric form at 4°C was used to initially suggest that the $(\alpha\beta)_2$ tetramer was the primary unit of spectrin *in situ*.[26] This suggestion was supported by flash photolysis cross-linking studies which indicated that spectrin on the membrane could be cross-linked only up to the tetrameric form,[27] the presence of spectrin tetramers on erythrocyte membranes incubated at physiological ionic strength and temperature,[28] and that the physiological amount of spectrin could be rebound to spectrin/actin-depleted vesicles only when added as the tetramer.[29] Larger oligomers of spectrin can be formed when highly concentrated spectrin heterodimers are incubated at 30°C, and some quantity of these high-order oligomers may be present on the membrane.[30] A biochemical and morphological analysis of oligomeric species of spectrin in a 0°C low ionic strength extract of red cell membranes, which included spectrin that was released from spectrin-4.1-actin complexes by mild urea treatment, indicated that spectrin tetramers, oligomers larger than tetramers, and dimers represent 45 to 55%, 25 to 35%, and 5 to 10% of the total spectrin, respectively.[31] If oligomers larger than tetramers represent 25 to 35% of total spectrin *in situ*, one would reasonably expect to see these oligomers in the recently published micrographs of spread, negatively stained intact membrane skeletons.[21,22] Paradoxically, oligomers of spectrin beyond the tetramer have not yet been visualized in these preparations,[21,22] leaving the purported significance of larger spectrin oligomers *in situ* unresolved.

Our current view of RBC spectrin morphology comes from rotary shadowed electron micrographs of spectrin isolated from low ionic strength extracts of red cell membranes.[32] Spectrin heterodimer sprayed onto mica in the presence of glycerol, dried, and shadowed at low angle with a platinum/carbon mixture appear as long flexible molecules consisting of two strands joined at their ends, with a contour length of ~100 nm and a thickness of 4 to 6 nm. Human RBC spectrin tetramer had a contour length of ~200 nm, suggesting that it was formed by end-to-end association of a pair of heterodimers without measurable overlap.[32] The inability to detect linear oligomers larger than tetramers in spectrin preparations was taken as circumstantial evidence for a head-to-head interaction. As both heterodimer strands appeared to be involved in tetramer formation, Shotton et al.[32] suggested that complementary noncovalent association sites exist at the junctional end of each heterodimer, and during tetramer formation, a reorientation of these association sites allows the α chain of one heterodimer to pair with the β chain of a adjacent heterodimer and vice versa. A drawing of the structure of the erythrocyte spectrin tetramer is shown in Figure 2.

Current knowledge of the detailed structure of the erythrocyte spectrin molecule has evolved in four stages: two-dimensional peptide mapping of the intact α and β subunits, cleavage of the spectrin molecule into chemical domains of a size that could be more easily analyzed, protein sequencing of several of the chemical domains, and nucleic acid sequence analysis of cDNA clones from erythroid α and β spectrin. The authors recommend a recent review by Speicher[33] as an excellent state-of-the-art discussion of spectrin structural analysis. Two-dimensional peptide mapping of human RBC spectrin α and β subunits radioiodinated in tyrosine residues indicated that the α and β subunits were distinct, demonstrating that the β subunit was not simply a smaller version of the α subunit.[34-37] The large spectrin subunits were subsequently dissected

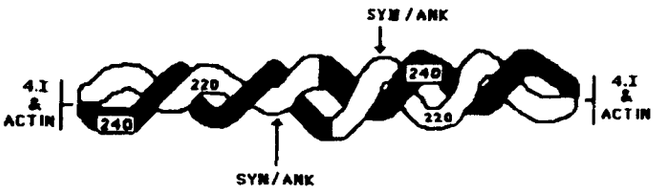
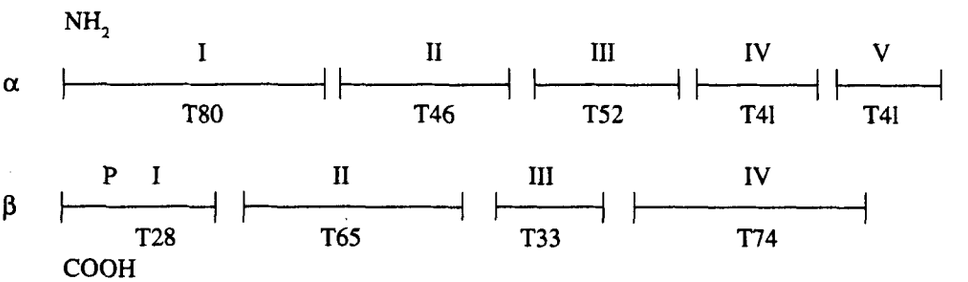


FIGURE 2. Model of the erythrocyte spectrin tetramer. The morphology of the 200-nm long erythrocyte spectrin tetramer is presented along with bivalent binding sites for syndein/ankyrin (SYN/ANK), protein 4.1 (4.1), and actin.

into more manageable pieces by mild tryptic digestion at 0°C which yielded nine relatively resistant chemical domains, five from the α subunit (α I to V) and four from the β subunit (β I to IV).[37,38] The chemical domains were separated on two-dimensional IEF-SDS-PAGE gels, and two-dimensional peptide mapping of the domains and overlapping fragments allowed the ordering of the domains into a linear domain map which is illustrated below. These illustrated chemical domains appear to account for 108 and 91% of the molecular weight of the α and β subunits, respectively. However, the molecular weight estimation of several of the domains based upon migration in polyacrylamide gels may be spurious.[33] There is a known loss of regions between the identified domains during mild proteolytic cleavage. Regions which are known to be proteolyzed to small peptides include the blocked N-termini of both subunits[39] and the phosphorylated 10-kDa segment of the β subunit.[38,40]



The spectrin β subunit is phosphorylated by a cAMP-independent protein kinase. The β subunit contains four phosphoamino acids (3 phosphoserine + 1 phosphothreonine) all clustered within 10 kDa of its C-terminal end.[40] The phosphorylated region of the spectrin β subunit is not localized within the trypsin-resistant domains because of its high susceptibility to proteolytic degradation. However, the phosphorylated C-terminal end must be adjacent to the β I domain because a 21-kDa phosphopeptide generated by NTCB cleavage of the β subunit shares partial sequence homology with β-I-tryptic peptides.[38] The blocked N-terminus of the α subunit which is lost upon mild tryptic digestion can be found about 10 amino acid residues before the N-terminal α I sequence.[33,41] Therefore the two subunits are oriented in an antiparallel manner with the N-termini at opposite ends of the dimeric molecule.

A more precise model of spectrin structure has come from exhaustive protein sequence analysis of spectrin chemical domains and recent nucleic acid sequence data. Speicher and colleagues have determined the protein sequence of 1435 amino acids of human RBC spectrin, or 35% of the total molecule.[33,41,42] The established protein sequence includes all of the α I domain,[41] all of the α II domain,[42] and partial sequences from peptides throughout the other chemical domains of the α and β subunits.[42] By analyzing the known protein sequence with a computer program which searches for internal repeats by sliding segments of sequence down

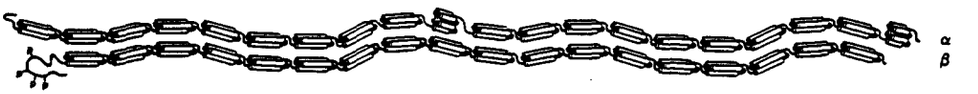


FIGURE 3. The triple helical repeat units of erythrocyte spectrin. The α and β subunits are composed of multiple triple helical segments connected by short nonhelical regions. The subunits are antiparallel with the N-terminus of the α subunit on the left. Each triple helical segment contains ~106 amino acids. The 10th segment in the α subunit is not homologous, and the 20th segment in this subunit may have a common evolutionary origin to the 10th repeat. The C-terminus of the β subunit contains four phosphorylation sites. (Reproduced from Speicher, D. W. and Marchesi, V. T., *Nature*, 311, 177, 1984. With permission, copyright © 1984 Macmillan Magazines Limited.)

the remainder of sequence, Speicher found that most of the α and β spectrin subunits are comprised of a 106 amino acid repeat unit with a molecular weight of ~12,000.[33,42] The repeat units are given arabic numbers beginning with the N-terminal end of the subunit (α-1 → α-20, β-1 → β-19), and homologous repeats contain 18 to 36% of their amino acids in common.[33,42] It has been suggested, based on protein sequencing, that all of the repeat units may be homologous except for the α-10, α-20, and β-19 repeats, which are nonhomologous and may have somewhat different lengths.[33,42] Predictions of spectrin conformation based upon computer analysis of spectrin sequence suggest that spectrin is comprised of linearly arranged triple helical structures (with two internal reverse turns), connected by flexible nonhelical regions (Figure 3).[42] Recent homology reinforcement conformational analysis has indicated that the repeat unit and the triple helical conformation unit are out of phase by approximately one half of the 106 amino acid repeat.[33] Based on the predicted spectrin heterodimer substructure (Figure 3), we can now consider how the condensed spectrin tetramer (~50 nm in length) seen on negative staining of the intact spectrin membrane skeleton (discussed in Section II.A) may be related to the 200-nm extended spectrin tetramer. One can envision the triple helical segments folding onto each other in an accordion-like fashion at the flexible regions, and new intramolecular associations being formed at physiological ionic strength, which are then broken under low ionic strength extraction conditions.

Recent nucleic acid sequence analysis of cDNA clones from erythroid α and β spectrin has been reported.[43-45] The results, discussed below, have confirmed and extended the protein sequencing data, been consistent with the 106 amino acid repeat unit being found throughout the α and β subunits, and has in one case corrected the placement of a 30 amino acid peptide fragment which had been assigned to the α-18 repeat unit.[42] Using an expression cDNA library derived from mouse anemic spleen, a cDNA clone with a 750-base pair (bp) insert has been identified, which directs the synthesis of a polypeptide in *Escherichia coli* that reacted with anti-mouse RBC spectrin IgG.[43] This cDNA clone was used to isolate the homologous human cDNA from a cDNA library derived from K562 erythroleukemic cells.[44] The nucleotide sequence of the human and mouse cDNAs partially overlap and span 1110 bp, with a total coding capability of 370 amino acids. Comparisons of the human- and mouse-deduced sequences with the known sequences of the human RBC spectrin α subunit[42] indicated that the sequence derived from the human cDNA clone spans a portion of the α-14 repeat unit, all of α-15 and α-16, and 5 amino acids in α-17. The mouse cDNA-deduced sequence overlapped the distal part of the human α-15 repeat unit, the α-16 repeat unit, and the proximal portion of the α-17 repeat unit and then encoded the remainder of the α-17 and the beginning of the α-18 repeat unit. Homology between the overlapping sequences between mouse and human α spectrin was 83%; the human sequence was in agreement with short segments in α-14 and α-15 which had been previously analyzed by protein sequence analysis,[42] and the mouse α-18-derived sequence indicated that a 30 amino acid segment had probably been misplaced within the α-18 repeat unit of human RBC spectrin.[42,44] Recently, Speicher suggested that his 30 amino acid segment may indeed be part

of the α-19 repeat unit.[33] A mouse spectrin β-subunit cDNA clone has been recently isolated and sequenced.[45] The deduced sequence indicated that it was homologous with the C-terminus of a peptide assigned to the β-8 repeat unit of human RBC spectrin,[42] then spanned all of the β-9 repeat unit and a portion of the β-10 repeat.[45] The derived sequence for mouse β-8, β-9, and β-10 repeat units contained most of the highly conserved amino acids of the 106 amino acid repeat unit of human α and β RBC spectrin except for a segment from residue 40 of β-9 to residue 30 of β-10, which may be located opposite to the nonhomologous α-10 repeat unit and therefore may have a different substructure from other β subunit repeat units.[45] Deduced amino acid sequences from a chicken smooth muscle α spectrin cDNA clone confirmed that the 106 amino acid repeat unit is preserved in distantly related nonerythroid spectrins and demonstrated a 20 to 36% overlap with the amino acid sequence of the human RBC spectrin α subunit.[46,47] Thus the presence of the 106 amino acid repeat unit, in a high molecular weight actin-binding protein, may be the best current criterion for including a protein within the spectrin family of molecules.[33]

Identification of functional sites along the erythrocyte spectrin molecule has been accomplished by three approaches: binding of spectrin-binding proteins to isolated spectrin subunits, rotary shadowing and electron microscopy of complexes of spectrin and spectrin binding proteins, and binding of spectrin-binding proteins to isolated chemical domains of RBC spectrin. Ankyrin, a high-affinity membrane-binding site for spectrin, binds to the β subunit.[48] Rotary-shadowed electron micrographs of the ankyrin-spectrin complex indicate that there are two potential ankyrin-binding sites per spectrin tetramer, each located 20 nm from the junction of the heterodimers (Figure 2).[49,50] This bilateral symmetry of binding sites supported the concept that the tetramer was formed by head-to-head interaction of two heterodimers. A 50-kDa NTCB fragment of the β subunit was demonstrated to contain the ankyrin-binding site.[51] Since the 50-kDa peptide overlapped the β I and β II spectrin domains,[51] this observation was in accord with the rotary shadowing and electron microscopy[49,50] and suggested that the physical ends of the strands visualized by electron microscopy represented the chemical ends of the individual subunits. Adjacent to the ankyrin-binding sites, but located at the N-terminus of the α subunit, is the 80-kDa α I domain which contains a binding site for the β subunit that is required for tetramer formation.[51] While the 10-kDa phosphorylated domain is found at the C-terminus of the β I domain,[37,40] phosphorylation does not appear to play a role in spectrin tetramer formation,[27] actin,[53] or ankyrin binding.[52] F-actin binds to both subunits of spectrin,[48,54] and rotary-shadowed electron micrographs indicated that the binding sites reside at the tails of the spectrin tetramer.[55] The weak interaction between spectrin and actin is strengthened by protein 4.1, which binds to both the α and β subunits[54] at a binding site which is indistinguishable from the location of the actin-binding sites when rotary-shadowed images are observed by electron microscopy.[49,50] It has been suggested that spectrin, protein 4.1, and f-actin form a ternary complex and that protein 4.1 and short actin protofilaments play the essential role of linking adjacent spectrin tetramers into the two-dimensional skeletal meshwork referred to as the membrane skeleton. It is not yet clear whether the protein 4.1 and actin-binding sites reside within the spectrin α V or β IV domains or, alternatively, within small fragments released from the N-terminus of the β subunit and C-terminus of the α subunit upon mild tryptic digestion of the spectrin molecule. As would be expected from the position of the actin- and protein 4.1-binding sites, their interactions are not affected by spectrin phosphorylation.[4,53] Indeed the functional importance of β-chain phosphorylation remains undetermined.

Structural changes have been detected in the N-terminal α I domain of human erythrocyte spectrin leading to abnormal tetramer formation in patients with HPP[56-58] and HE.[59,60] A defect at the tails of the spectrin tetramer causing an abnormal spectrin-4.1-actin ternary complex is found in some patients with HS.[61,62] These studies clearly demonstrate the importance of spectrin tetramer formation and ternary complex formation to the structural stability of the membrane skeleton and maintenance of normal biconcave shape.

b. Actin

Actin was first reported in 1962 to occur in the human erythrocyte membrane.[63] Subsequent studies indicated that erythrocyte actin was equivalent to the "band 5" polypeptide visualized on SDS-PAGE of RBC membranes, could polymerize into the typical double helical filaments which could be decorated by myosin head groups, and stimulated myosin ATPase in a manner similar to skeletal muscle actin.[64-66] A comparison of the polymerization kinetics of erythrocyte and muscle actins indicated that at optimal ionic conditions the rate and final extent of polymerization were reduced for erythrocyte actin.[67] This functional difference is perhaps related to the fact that erythrocyte actin is present as the β isoform, rather than as the α isoform found in muscle cells or as a mixture of isoelectric variants (β and γ) found in other cells.[68] The authors recommend two excellent reviews on general aspects of actin structure, polymerization, and interactions which are beyond the scope of this review.[69,70]

Erythrocyte spectrin dimers and tetramers bind to f-actin, but not g-actin.[53] While both oligomeric forms of spectrin bind to actin, only the bivalent tetramer can cross-link f-actin solutions, causing increases in viscosity.[53] The interaction of RBC spectrin with actin is quite weak with a K_A at 20°C of $5 \times 10^3\ M^{-1}$.[71] Protein 4.1, which is discussed in Section II.B.1.c, strengthens the spectrin-actin interaction, possibly by formation of a ternary complex. The individual α and β subunits of spectrin, when separated by treatment with urea or SDS and subsequently isolated, cannot bind to f-actin.[48,54] αβ Heterodimers formed by recombination of the isolated subunits can bind f-actin, leading to the suggestion that both subunits are required for actin binding.[54] Rotary shadowing and electron microscopy have indicated that actin binds to the tail end of spectrin, with dimers capable of binding one actin filament, and tetramers capable of binding two.[55] As would be predicted, f-actin binding to spectrin/actin-depleted-inverted erythrocyte vesicles is stimulated by reconstitution of vesicles with spectrin.[72] Tetramers are twice as effective as dimers in stimulating actin binding,[72] and these spectrin-reconstituted vesicles are capable of inducing large increases in the viscosity of actin solutions.[73]

The spectrin-4.1-actin complex (or high molecular weight complex [HMW complex]) isolated by low ionic strength extraction of erythrocyte membranes was found to accelerate actin polymerization[74,75] by decreasing the lag phase of polymerization.[76] Neither spectrin nor protein 4.1 alone could stimulate actin polymerization, and polymerization was blocked by cytochalasins which bind to the barbed (fast-growing) end of actin filaments.[74,75] These findings led to the concept that actin on the erythrocyte membrane and in the HMW complex is present as short oligomers which serve as nucleation sites for actin polymerization.[74,75] Since there are 500,000 copies of actin per erythrocyte membrane[77] and 3 to 4×10^4 high-affinity binding sites for cytochalasin,[78] it has been calculated that the average length of actin protofilaments on the membrane is 12 to 17 actin monomers.[77] Remarkably, actin profilaments observed on electron microscopy of negatively stained intact membrane skeletons fall within a narrow range of lengths, with a mean length of 33 to 37 nm, equivalent to a double stranded helix with 12 to 14 actin monomers.[21,22] Based on the fact that spectrin-4.1-actin complex stimulation of actin polymerization is blocked by cytochalasin E, Pinder et al.[77,79] have concluded that spectrin and 4.1 bind to and cap the pointed (slow-growing) end of actin filaments. Similarly, Cohen and Branton described that the orientation of newly polymerized actin on spectrin-reconstituted-inverted erythrocyte membrane vesicles had the pointed ends facing the membrane exclusively,[80] an orientation which was opposite to the interactions of actin with most cell membranes. Unfortunately, these studies appear to have been conducted at g-actin concentrations below the critical concentration for the pointed end. Subsequent studies indicated that, at higher concentrations of g-actin, spectrin-4.1-actin complexes in solution[81] and on the membrane[82] are capable of extension at both the pointed (slow-growing) and barbed (fast-growing) end. The electron micrographs of actin protofilaments in the native membrane skeleton[21,22] indicated that they contained 4 to 8 spectrin (and 4.1) molecules attached to the ends and center of the protofilament.

Therefore, the spectrin-4.1 complex is bound to both the barbed and pointed ends of the protofilaments and presumably stabilizes both ends against depolymerization.[76,80]

An important unanswered question is what regulates the length of actin protofilaments on the erythrocyte membrane? Erythrocyte tropomyosin (discussed in detail in Section II.B.1.e) binds to f-actin at a stoichiometry of 1 tropomyosin per 6 to 7 monomers and appears to be capable of head-to-tail self-association within the grooves of the double helix.[83] Tropomyosin may strengthen the erythrocyte actin protofilament, protecting it from breakage and erosion of g-actin monomers.[84] Therefore, while a protofilament of 13 monomers would be protected by 2 tropomyosin filaments lying within each groove, a protofilament of 17 monomers would have 4 monomers susceptible to breakage or erosion. While this concept may explain why RBC actin protofilament should be composed of a multiple of 13 monomers, it does not explain why the length should be 13 monomers rather than 26 or 39. Another interesting, but virtually untouched question concerns the characteristics of the spectrin-binding domain of erythrocyte actin. These questions should keep students of the field occupied for several years to come.

c. Protein 4.1

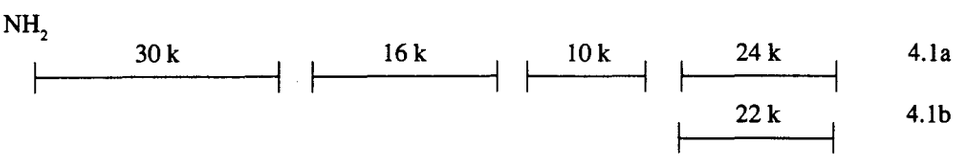
Protein 4.1 is a peripheral membrane protein which is localized on the cytoplasmic surface of the erythrocyte membrane.[24] The nomenclature "4.1" indicates that this polypeptide migrates slightly more rapidly than erythrocyte band 3 (the anion channel) as a single band on SDS-PAGE in a continuous buffer system.[85] The apparent molecular weight for human erythrocyte protein 4.1 determined in this gel system was ~78 kDa, and based upon its relative protein dye staining, it was calculated to be present in ~200,000 copies per erythrocyte.[24]

Interestingly, protein 4.1 could be resolved into two distinct polypeptides (80 and 78 kDa) when erythrocyte membrane proteins were separated by SDS-PAGE in a discontinuous buffer system,[86] and these two polypeptides are referred to as proteins 4.1a and 4.1b.[16] We have demonstrated that proteins 4.1a and 4.1b are present in approximately equal amounts (ratio 4.1a/4.1b = 1.0 to 1.2) in human erythrocyte ghosts with the 4.1a isomorph predominating in the Triton membrane skeleton (4.1a/4.1b = 1.2 to 2.0).[16,87] Proteins 4.1a and 4.1b are both labeled to an equal extent when intact erythrocytes were labeled with [^{32}P]-orthophosphoric acid, and therefore both are phosphoproteins.[16] One-dimensional peptide maps of ^{32}P-labeled 4.1a and 4.1b and two-dimensional peptide maps of ^{125}I-labeled 4.1a and 4.1b both yielded identical peptide patterns for the 4.1 isomorphs.[16] These results indicated that 4.1a and 4.1b are sequence-related phosphoproteins and raised the question of what characteristic distinguishes the isomorphs? As pointed out previously,[16] the different migration of 4.1a and 4.1b on discontinuous SDS-PAGE could be due to (1) the proteolytic cleavage of an ~2-kDa terminal end of 4.1a yielding 4.1b, (2) a difference in phosphate content yielding a charge difference, or (3) some other posttranslational modification (e.g., carboxy-methylation) leading to a charge differential. Several pieces of more recent information give some insight into these possibilities. Our data, which indicated that erythrocytes metabolically labeled with radioactive phosphate incorporate equal amounts of phosphate into 4.1a and 4.1b, would argue against phosphorylation being the distinguishing characteristic.[16] This finding is supported by the observation that the distinguishing characteristic resides within an acidic nonphosphorylated 22- to 24-kDa C-terminal domain of protein 4.1 (a + b).[88] Two observations bear upon the possibility that the difference between 4.1a and 4.1b is due to proteolytic cleavage. Proteins 4.1a and 4.1b represent 90% of purified human RBC protein 4.1 preparations; the remaining protein includes 87-, 85-, and 67-kDa polypeptides.[89] The 87-, 85-, and 67-kDa polypeptides all cross-react with 4.1 antibodies,[89] and the 87- and 85-kDa polypeptides yield identical two-dimensional iodopeptide maps to protein 4.1a (80 kDa) and protein 4.1b (78 kD),[89] while the 67-kDa peptide yielded an iodopeptide map which was a subset of the peptides generated from 4.1a and 4.1b.[89,90] Therefore, if the distinction between the 87-, 85-, 80-, and 78-kDa 4.1 isomorphs is based on consecutive cleavage of terminal fragments allowing the following conversion, 87 → 85 → 80 → 78 kDa, then the fact

that their iodopeptide maps appear identical would suggest that the 9 kDa of cleaved terminal regions must contain no iodinatable tyrosine residues.[89] The sequence of human erythrocyte protein 4.1 cDNA indicates a 588 amino acid 4.1 molecule with a calculated molecular mass of 66,303 and predicted N-terminal sequence which is identical to the known protein sequence of residues 1 to 42 of 4.1 (a + b).[91] This suggests that the protein is not translated as an 87-kDa species which posttranslationally is proteolytically cut to an 80-kDa (4.1a) species. It is possible that the difference between the 87/85-kDa polypeptides and 4.1a/4.1b could be due to posttranslational modifications (e.g., carboxy-methylation), while the conversion of 4.1a to 4.1b could be proteolytic. Since the derived sequence of the C-terminal 20 amino acids of protein 4.1 contains no tyrosine residues, a proteolytic cleavage of this segment would yield identical peptide maps for 4.1a and 4.1b. A finding which could be used to argue against a proteolytic conversion of 4.1a to 4.1b is that in mouse red cells, where erythropoiesis was inhibited and the red cell population uniformly aged by serial hypertransfusion, the 4.1a/4.1b ratio increased as the cells aged.[92] Because the total 4.1 content did not change with red cell age, it would appear that 4.1b was converted to 4.1a by a posttranslational modification other than proteolytic cleavage.[92] While the study by Mueller et al.[92] does not support a proteolytic conversion of 4.1a to 4.1b, it does not rule out a proteolytic conversion of 4.1b to 4.1a. Because the C-terminal 20 amino acid stretch of protein 4.1 from residues 569 to 588 is quite acidic (5 acidic amino acids and only 1 basic amino acid),[91] its cleavage could cause a decrease in SDS binding sufficient to cause retardation of electrophoretic mobility. In other words, 4.1a could be a cleaved form of 4.1b with anomalous migration in SDS-PAGE. While the structural difference between 4.1a and 4.1b is still unresolved as just explained, the presence of 87- and 85-kDa isomorphs of 4.1 at low concentrations (<10% of total 4.1) in the human erythrocyte cannot be explained by proteolytic processing. Therefore the distinction between the 87/85-kDa polypeptides and 4.1a + 4.1b could be that the large isomorphs are (1) a dephosphorylated form of protein 4.1a + 4.1b or (2) a carboxy-methylated form[93] since both of these posttranslational modifications would be expected to slow the migration of 4.1 isomorphs on SDS-PAGE. It may also be significant that carboxy methylation of protein 4.1 increases tenfold as red cells age,[94] in parallel with increases in the 4.1a/4.1b ratio. It will be interesting to determine whether the content of the 87- and 85-kDa isomorphs also increase with red cell age. In addition to the structural differences between 4.1 isomorphs remaining unclear, it is also not clear whether they have any functional distinctions. Proteins 4.1a and 4.1b both have equal ability to bind to spectrin[16] and to their high-affinity membrane attachment site,[95] while the functional capabilities of the 87-, 85-, and 67-kDa protein 4.1 isomorphs have not been adequately explored.

Protein 4.1 isolated by high ionic strength extraction of spectrin/actin-depleted erythrocyte vesicles[49,50] or Triton membrane skeletons[96] is a monomer which is capable of time-dependent oligomerization in solution. Amino acid composition analysis indicates that protein 4.1 contains 33% of hydrophobic amino acids[50] and a hydrophobicity index equal = 1020, indicating moderate hydrophobicity.[96] Its hydrodynamic properties have yielded an $S_{20,w}$ value = 3.5, a frictional ratio = 1.80, an axial ratio = 9:1, a Stokes radius = 4.15 nm, and a calculated molecular weight = 78 kDa.[96] These properties suggest a protein that is an appreciably asymmetric prolate ellipsoid.

A structural map of protein 4.1 has been obtained by mild chymotryptic cleavage of the protein at three major sites. The domains have been placed in the sequence shown here based upon peptide mapping of overlapping NTCB fragments.[88]



The 30-kDa N-terminal domain of protein 4.1 is basic and is thought to contain the membrane attachment site;[97] the 16- and 10-kDa polar domains contain the phosphorylation sites (discussed later);[98] and the acidic 22/24-kDa C-terminal domain contains the region of variability between the a and b isomorphs.[88] As introduced earlier, 4.1 cDNA derived from a human reticulocyte cDNA library has been cloned and sequenced.[91] A single, long open reading frame in the cDNA encoded a 588 amino acid 4.1 molecule with a calculated molecular mass of 66,303 Da. Amino acids 1 to 42 of the predicted sequence were identical to the amino terminus of erythrocyte protein 4.1 as determined by protein sequencing, and amino acids 405 to 471 of the predicted sequence are identical to the previously determined protein sequence for the "10-kDa" domain.[91,99] The 30-kDa membrane-binding domain of protein 4.1 is very hydrophobic in sequence, contains all seven cysteine residues in the molecule, thus explaining its susceptibility to NTCB cleavage, and has a predicted secondary structure which contains substantial β-sheet structure in addition to amphipathic α helices.[91] The 16-kDa domain is hydrophilic, with an unusually high proline content (9.3%) and a largely α-helical-predicted secondary structure. The 10-kDa domain is highly charged (46% asp, glu, arg, lys, his) with a long α-helix as its most prominent feature.[91] The 22/24-kDa domain is acidic and only contains 117 amino acids, with a calculated size of 12,636 Da. Therefore the apparent size of 22/24 kDa in SDS-PAGE is anomalous, and this explains the difference between the apparent molecular weight for 4.1 calculated from migration on SDS-PAGE (78/80 kDa) and the calculated molecular weight based upon cDNA sequencing (66,303 Da). The elegant work of Conboy et al.[91] described previously has given us an extremely detailed knowledge of the erythrocyte protein 4.1 molecule and will allow comparisons with nonerythroid 4.1 molecules from various sources.

Protein 4.1 can serve as a substrate for a number of protein kinases, including a cAMP-independent and cAMP-dependent protein kinase[98,100,101] and protein kinase C.[98,102-106] Physiological activation of protein kinase C appears to be mediated by arachidonic acid-containing diacylglycerol which is produced by a hormonally induced breakdown of polyphosphoinositides.[107] The tumor-producing phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) and 12-0-tetradecanoyl phorbol 13-acetate (TPA), stimulate the phosphorylation of protein 4.1 *in vivo* and *in vitro*,[98,102-106] presumably by recruiting cytoplasmic protein kinase C to the cytoplasmic surface of the erythrocyte plasma membrane.[104,105] Diacylglycerol added exogenously or endogenously (by phospholipase C action) to intact erythrocytes also, as would be anticipated, stimulated the phosphorylation of protein 4.1.[103] The extent of phosphorylation of protein 4.1 by cAMP-dependent protein kinase and protein kinase C in the intact erythrocyte has been estimated to be 0.6 mol P_i/mol 4.1 (cAMP-PK)[106] and 1.5 to 4.0 mol P_i/mol 4.1 (PK-C),[103,106] with ~80% phosphorylation of serine residues and ~20% threonine residues for both enzymes.[106] The protein kinase C phosphorylation sites on erythrocyte protein 4.1 have been localized to the 16-kDa domain, while the cAMP-dependent kinase sites are located in the 10- and 16-kDa domains.[98] As is discussed in detail later, since the 10-kDa domain has been demonstrated to contain the spectrin/actin-binding site in protein 4.1, the role of phosphorylation in regulating this interaction has become a subject of great interest.

Purified human RBC protein 4.1 binds saturably to human RBC spectrin dimer in solution, with a k_D value of 100 to 250 n*M* when determined by an immunoprecipitation assay,[50,61,62] 2000 n*M* when determined by a lengthy and low-resolution rate zonal sedimentation of spectrin-4.1 complex,[108] and ~12 n*M* when measured by fluorescence resonance energy transfer.[109] The maximal binding capacity measured in solution is 2 mol 4.1/mol spectrin dimer[61,62,50,108] and probably indicates a dimerization *in vitro* which, if it occurred *in vivo*, would leave one half of the 200,000 binding sites for 4.1 on the erythrocyte spectrin molecules unoccupied. Based on a K_a of $8.6 \times 10^7\ M^{-1}$ measured by fluorescence resonance energy transfer, an association free energy of –10.8 kcal/mol ($\Delta G_A = -RT \ln K_a$) has been calculated.[108] As the enthalpy was determined to be –6 kcal/mol by microcalorimetry, the entropic contribution to the spectrin-protein 4.1 interaction ($-T\Delta S$) can be calculated to be –4.8 kcal/mol ($\Delta G = \Delta H - T\Delta S$).[109] This

suggested that the protein environment participates in the spectrin-4.1 complex, and it was found that proton uptake is involved in the spectrin dimer-band 4.1 complex as follows:

$$[\text{spectrin}] + [\text{protein 4.1}] \underset{-H^+}{\overset{H^+}{\rightleftharpoons}} [\text{spectrin-4.1-}H^+]$$

Protein 4.1 is capable of binding weakly to the individual α and β subunits of human RBC spectrin in solution,[54] yet recent results have suggested that the α and β subunits of avian RBC spectrin are incapable of individually forming a protein 4.1-dependent complex with actin and will do so only when reconstituted into the original αβ complex.[110] Furthermore, this study demonstrated that, with avian spectrins which have a common α subunit, the ability of spectrins to bind protein 4.1 is determined by the β subunit since RBC spectrin and brain spectrin(240/235) were capable of binding protein 4.1, while TW 260/240 could not.[110] Rotary shadowing and electron microscopy of the human RBC spectrin-4.1 complex indicates that protein 4.1 binds to the tails of the spectrin tetramer (Figure 2), a site which is indistinguishable at this level of resolution from the actin protofilament-binding site.[49,50] Protein 4.1 has the ability to strengthen the spectrin-actin interaction, as demonstrated by a sedimentation assay[111] and low shear viscometry.[112] The addition of protein 4.1 to solutions of spectrin tetramers and f-actin gives the resulting gel the ability to reform after shearing (termed thixiotropy).[113] In the presence of human RBC protein 4.1, the weak spectrin dimer-f-actin interaction ($K_D = 2 \times 10^{-4} M$) is increased in affinity by almost eight orders of magnitude ($K_D = 1 \times 10^{-12} M$).[71] As would be expected, the addition of 4.1 to inside-out vesicles which have been previously depleted of protein 4.1 has a stimulatory effect upon the ability of reconstituted spectrin to bind f-actin.[114] An 8-kDa fragment of human RBC protein 4.1 found within the 10-kDa domain possesses activity comparable to intact protein 4.1 in stimulating the spectrin-actin interaction.[115] This 8-kDa fragment of protein 4.1 has been sequenced and found to be highly charged (31 of 67 residues), suggesting that this entire segment may be exposed on the surface of the molecule.[99]

To summarize, protein 4.1 binds to both ends of the spectrin tetramer, strengthening the binding of spectrin to actin protofilaments and allowing the extensive cross-linking observed on electron microscopy of the intact membrane skeleton. Protein 4.1 could stimulate the binding of spectrin to actin by either of two plausible mechanisms: (1) it could bind to spectrin at a site near the actin-binding site, causing an allosteric change in the spectrin molecule which would promote actin binding; or (2) a spectrin-protein 4.1-actin ternary complex could be formed. While most workers in the field seem to prefer the concept of a ternary complex, there is currently little credible evidence that protein 4.1 can enter into a binary complex with actin in the absence of spectrin. Yet we know that the binding sites for spectrin and actin must be very close to each other on the 4.1 molecule since both are contained within a 67 amino acid segment of the 10-kDa domain.

There has recently been substantial interest in the regulation of the spectrin-protein 4.1-actin interaction. Eder et al.[108] demonstrated that protein 4.1, which is phosphorylated in two sites by a purified cAMP-independent protein kinase, has its affinity for RBC spectrin reduced almost fivefold (K_D of $2 \times 10^{-6} M$ to a K_D of $9.4 \times 10^{-6} M$). This interesting result suggests that the binding of protein 4.1 to spectrin may be down-regulated by phosphorylation, but this is limited by the fact that the affinities measured by separation of spectrin-4.1 complexes from noncomplexed proteins by sedimentation through sucrose gradients[107] is one to two orders of magnitude weaker than the affinity measured by other techniques[50,61,62,109] and that the two phosphorylation sites have not been demonstrated by these authors to be within the 10-kDa spectrin-binding domain of protein 4.1. If we accept the K_D values for binding of phosphorylated ($9.4 \times 10^{-6} M$) and nonphosphorylated ($2 \times 10^{-6} M$) protein 4.1 to spectrin that were reported by Eder et al.,[108] then we still must recognize that such a shift in K_D would cause only a small modulation of the

spectrin-4.1 interaction on the erythrocyte membrane where the protein 4.1 concentration is in excess of 400 m*M* (based on the previously calculated concentration of spectrin on the membrane surface).[30] An interesting question will be the determination of whether protein 4.1, which is phosphorylated in the 10- and 16-kDa domains by cAMP-dependent protein kinase and the 16-kDa domain by protein kinase C, shows more pronounced decreases in spectrin affinity.

A second possible mechanism of regulating the spectrin-4.1-actin interaction is through the binding of calmodulin to spectrin. Calmodulin binds weakly to erythrocyte spectrin under nondenaturing conditions, with a K_D of 6.7 to 25 μM.[116-118] A 10-kDa CNBr-derived peptide of RBC spectrin accounts for the calmodulin interaction with spectrin under denaturing conditions (6 *M* urea).[119] This peptide is derived from the N-terminus of the β subunit within the β IV domain, at a site close to the protein 4.1- and actin-binding sites.[119] Photolysis of ^{125}I-labeled azidocalmodulin bound to spectrin under nondenaturing conditions led to a labeling of the β subunit that was stimulated by calcium and inhibited by underivatized calmodulin or the calmodulin inhibitor trifluoperazine.[120] In this same study, underivatized calmodulin in the presence of Ca^{2+} was demonstrated to inhibit the protein 4.1-stimulated cosedimentation of RBC spectrin and actin.[119] These data taken together indicate that calcium and calmodulin may regulate the spectrin-actin interactions via a calcium-dependent binding of calmodulin to the C-terminal end of the spectrin β subunit. It has been estimated, based on the concentration of calmodulin and calcium in the erythrocyte and the calculated K_D values for the calmodulin-spectrin and Ca^{2+}-calmodulin interactions, that only ~5% of the RBC spectrin molecules should contain bound calmodulin *in vivo*, a figure which may rise to 30% under pathological conditions.[118] Therefore, while calmodulin binding may suppress the spectrin-4.1-actin interaction in a small subset of ternary complexes *in vivo*, it should not be considered a Ca^{2+}-dependent on-off switch for the entire membrane skeleton.

d. Protein 4.9

Protein 4.9 is one of the core membrane skeletal proteins which remains after high ionic strength extraction of Triton shells and as such probably plays an important role in this structure. This protein, which is present in 100,000 copies per erythrocyte, is composed of a single 48-kDa polypeptide on SDS-PAGE. Protein 4.9 has been purified and found to be a trimer in solution with an apparent molecular weight of 145 kDa and a Stokes radius of 5 nm, as determined by gel filtration and cross-linking by copper-phenathroline catalyzed oxidation.[121] Siegel and Branton[121] found that protein 4.9 does not bind spectrin in solution, but is capable of bundling actin filaments *in vitro*. While the proof that 4.9 serves as an actin-bundling protein *in vitro* is quite compelling, negative-stained images of the intact membrane skeletons have not as yet demonstrated the presence of truncated or long actin bundles.[21,22] Therefore we are left with the question of whether protein 4.9 may play some other, as yet unidentified, function within the membrane skeleton.

Protein 4.9 is phosphorylated *in vivo* and *in vitro* by a cAMP-dependent protein kinase and protein kinase C.[100,103-106] In the presence of TPA, protein kinase C causes incorporation of 1.2 mol P_i/mol protein 4.9 in intact erythrocytes, while cAMP-dependent protein kinase causes incorporation of 3.4 mol P_i/mol protein 4.9.[108] Of the phosphate incorporation caused by either kinase, 74 to 79% is bound to serine and 21 to 25% to threonine. The location of the phosphorylation sites within the protein 4.9 molecule and their function(s) remain unknown.

e. Tropomyosin

When erythrocyte ghosts are prepared in the presence of Mg^{2+}, tropomyosin remains with the membrane fraction in ~67,000 copies per erythrocyte.[83] Erythrocyte tropomyosin is composed of two polypeptides of 29 and 27 kDa, and chemical cross-linking with an amino-reactive bifunctional reagent indicated that, as expected for a nonmuscle tropomyosin, the erythrocyte protein is a dimer composed of two equivalent 29-kDa polypeptides or two equivalent 27-kDa

polypeptides. As the ratio of 29- to 27-kDa polypeptides is 3:1, we would anticipate that 29/29-kDa homodimer would be the prevalent form of tropomyosin *in situ*, but it has not yet been determined whether 29/27-kDa heterodimers also exist on the membrane. Erythrocyte tropomyosin has a Stokes radius = 5.9 nm, a $S_{20,w}$ = 2.5, and a partial specific volume = 0.72 cm^3/g, based upon its amino acid composition.[83] Based upon these parameters, a molecular weight of 60,000 and a frictional ratio of 2.07 have been calculated, indicating a highly asymmetric dimer. RBC tropomyosin binds to f-actin with a stoichiometry of one tropomyosin dimer per 6 to 7 actin monomers, and an affinity which depends upon the Mg^{2+} concentration (K_D = 385 n*M* at 2 m*M* Mg^{2+}, K_D = 34.5 n*M* at 10 m*M* Mg^{2+}).[83] It has been calculated that there is one tropomyosin dimer per 7 to 8 actin monomers in the human erythrocyte.[92] This number of tropomyosin molecules would be sufficient to occupy both grooves of ~87% of the 13 actin monomer protofilaments within the erythrocyte membrane skeleton.

As discussed in the previous section on actin, it is quite possible that erythrocyte tropomyosin, from its position within the grooves of the actin protofilament, serves to stabilize the actin filament, as well as defining its length as an integral of 13 monomers by protecting against monomer erosion. It has also been suggested, based upon preliminary experiments, that tropomyosin once bound to f-actin can partially block the binding of RBC spectrin to the filament.[122] Furthermore, based on this preliminary study, it was suggested that the actin protofilaments might have spectrin bound only to the ends since the center of the filament would be blocked by tropomyosin.[122] However, recent electron micrographs of intact membrane skeletons prepared in the presence of Mg^{2+} (and therefore containing tropomyosin) demonstrated spectrin molecules bound to the center as well as the ends of actin protofilaments.[21] Therefore, further work is required to determine whether tropomyosin may regulate the spectrin-actin interaction *in vivo*.

Erythrocytes contain 2400 to 6000 copies of myosin, approximately 20 to 40% of which is bound to the membrane.[123,124] RBC myosin is an authentic vertebrate myosin with two globular heads at the end of a rod-like tail of ~150 nm as viewed by rotary shadowing and electron microscopy.[123] At low ionic strength, the myosin forms short bipolar filaments which are 0.3 to 0.4 μm long.[123,124] Erythrocyte myosin contains a 200-kDa heavy chain and two light chains of 25 and 19.5 kDa which occur in a 1:1:1 molar ratio.[123,124] The RBC myosin has ATPase activities which are similar to many nonmuscle myosins in being calcium activated and magnesium inhibited, as well as being activated in 0.5 *M* KCl in the absence of divalent cations.[123,124] The Mg^{2+}-ATPase activity of myosin is not stimulated by actin until phosphorylated in a single site on the 19.5-kDa light chain by myosin light chain kinase, which results in a sixfold stimulation of the actomyosin Mg^{2+}-ATPase activity. A very provocative model for the role of actin and myosin in regulating red cell shape has appeared.[84] In this model, actin protofilaments are attached to the cytoplasmic surface of the membrane via their barbed ends. In the region of the dimple in the biconcave erythrocyte, very long actin filaments, originating from opposite sides of the cell, would interact with a population of 300-nm long bipolar myosin filaments. The myosin filaments would then draw the two sides of the membrane together, forming the dimples of the biconcave RBC. While fascinating, this model has several serious drawbacks. Actin protofilaments viewed on negative staining of intact RBC membrane skeletons have spectrin and 4.1 bound at both ends[21,22] and therefore are not jutting off the membrane like a cluster of grapes attached at one end. The distance across the dimple region of the red cell (810 nm) and the length of the myosin filament (300 nm) would indicate that the actin filaments in the dimple region would have to be >250 nm long to interact with the myosin filaments. Actin filaments of this length are not observed on electron microscopy of the erythrocyte membrane skeleton.[21,22] Based on the value of 6000 myosin molecules per cell, 30% of which are membrane bound, and myosin filaments of 300 nm in length (30 myosin molecules), one can calculate that there would only be 60 membrane-bound myosin filaments per red cell. The scarcity of membrane-associated myosin filaments raises the question of whether myosin in the mature erythrocyte is

simply a remnant from earlier stages of erythropoiesis. However, it is quite likely that tropomyosin may play the important myosin-independent physiological roles which were described earlier.

2. Linking Proteins

In the previous sections, we described the structure and functional interactions of the proteins which form the core of the membrane skeleton. In this section, we acquaint the reader with the proteins which serve to link the skeletal proteins to the bilayer.

a. Ankyrin

There is no better understood interaction of a cytoskeletal protein with a membrane surface than the well-characterized spectrin-ankyrin-band 3 interaction. In this section, we focus on the discovery, structural analysis, and spectrin-binding capability of ankyrin. In Section II.B.3.a, we will discuss the ankyrin-band 3 interaction in detail.

Our understanding of the interaction of RBC spectrin with the cytoplasmic surface of the erythrocyte membrane began with the establishment of a quantitative *in vitro* assay. Bennett and Branton[125] found that a ^{32}P-labeled spectrin heterodimer would reassociate with spectrin/actin-depleted-inverted erythrocyte vesicles in a saturable, pH- and salt-dependent manner. Several laboratories, using modifications of this basic assay, determined a K_D value of 16 to 50 n*M* for the interaction of spectrin dimer with the inverted vesicles, and a maximal binding capacity of 116 to 177 μg spectrin dimer per milligram of vesicle protein.[29,34,61,126-129] The key to discovering the identity of the high-affinity spectrin-binding site on the cytoplasmic membrane surface was the demonstration by Bennett[126] that mild chymotryptic digestion of the spectrin/actin-depleted vesicles destroyed spectrin-binding capability and released an active fragment of the binding protein. From the chymotryptic water-soluble digest, a 72-kDa fragment was isolated which would competitively inhibit the binding of ^{32}P-spectrin to depleted inverted vesicles with a K_I = 100 to 180 n*M*[34,126] and would form a 1:1 mol/mol complex with spectrin dimer in solution.[126] Once the 72-kDa fragment was identified as a portion of the high-affinity binding site, then two complimentary methodologies were used to demonstrate its parent molecule. Bennett and Stenbuck[130] prepared antibodies against the 72-kDa polypeptide and then used these antibodies to specifically immunoprecipitate proteins 2.1 and 2.2 from Triton X-100-solubilized inside-out erythrocyte vesicles. Yu and Goodman[34] and Luna et al.[36] performed peptide-mapping analysis on the 72-kDa fragment and all of the major erythrocyte membrane proteins. Comparison of these peptide maps indicated that the 72-kDa fragment had been generated from the family of sequence related proteins 2.1, 2.2, 2.3, and 2.6.[34,36] Both methodologies led to the same conclusion, and the spectrin-binding protein(s) were named ankyrin[130] and syndeins.[34] The ankyrin terminology is used by most workers in the field and is used in this review.

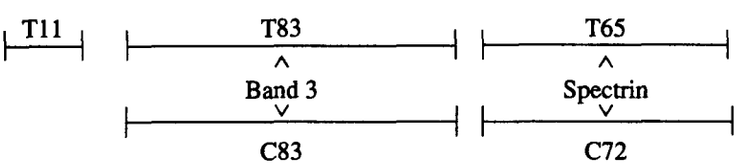
Proteins 2.2, 2.3, and 2.6 are proteolytic fragments of the progenitor polypeptide protein 2.1.[34,36,131] A membrane-associated serine protease cleaves protein 2.1 in consecutive steps (2.1 → 2.2 → 2.3 → 2.6 → 3′) down to a membrane-associated 89-kDa spectrin-binding fragment referred as 3′.[131] A Ca^{2+}-dependent membrane-associated protease cleaves protein 2.1 to 2.3, followed by the serine protease cleavage of 2.3 → 2.6 → 3′.[131] Since all of the ankyrin isomorphs are present, even when artifactual proteolysis is avoided by extensive use of a wide spectrum of protease inhibitors, it is likely that all are present *in vivo*.[131] The early studies on reassociation of spectrin dimers with spectrin-depleted-inverted vesicles indicated a maximal binding capacity (116 to 177 μg spectrin per milligram vesicle protein) which was one half of the spectrin content on the native membrane (250 μg spectrin per milligram ghost protein or 330 μg spectrin per milligram vesicle protein). This suggested that, if the binding sites were not being destroyed during vesicle preparation, the number of ankyrin molecules capable of binding spectrin must be one half of the number of spectrin dimer copies.[34] To test this suggestion, Goodman and Weidner[29] compared the binding of spectrin tetramers and dimers to spectrin/actin-depleted

vesicles. We found that, while dimers and tetramers bound to ankyrin *in situ* with equal affinity (K_D = 17 n*M*) close to the physiological concentration of spectrin could be reassociated in the form of a tetramer (270 μg spectrin per milligram vesicle protein), only one half could be rebound as a dimer (132 μg spectrin per milligram vesicle protein).[29] This study demonstrated that even though each tetramer should be capable of binding two ankyrin molecules *in situ* the stoichiometry is one ankyrin molecule per spectrin tetramer.[29] The reason is that the number of copies of ankyrin on the membrane (100,000)[29,34,126,132] is equal to the number of copies of spectrin tetramer (Table 1).

Ankyrin has been isolated by high ionic strength extraction of spectrin/actin-depleted vesicles[49,50] or Triton membrane skeletons,[133] indicating that it falls into the category of a peripheral membrane protein. The purified protein has a Stokes radius = 58 Å determined by gel filtration, a $S_{20,w}$ = 6.9 by rate zonal sedimentation through a sucrose gradient, a partial specific volume (v) = 0.73 cm^3/g based on amino acid composition, a frictional ratio f/fo = 1.46 indicative of a somewhat asymmetric globular protein, and a calculated molecular weight = 169,000 Da. The apparent molecular weight determined by migration on SDS-PAGE is ~200,000 Da. Therefore, precise molecular weight must await the sequencing of ankyrin.

Purified RBC ankyrin binds to spectrin dimer and tetramer in solution with a K_D of 100 n*M* and a stoichiometry of one ankyrin per spectrin heterodimer (two per tetramer) when analyzed by an immunoprecipitation assay.[50] Ankyrin binds to the spectrin β subunit[48] at a site which is 20 nm from the junctional end of the heterodimer (Figure 2) as determined by rotary shadowing and electron microscopy.[50] Since there is bilateral symmetry to the spectrin tetramer, two ankyrin molecules bind at sites which are separated by 40 nm.[50] Binding studies of purified RBC ankyrin and spectrin dimers which have been analyzed by nondenaturing gel electrophoresis have led to inflated K_D values (1.3 to 2.5 μ*M*) and the conclusion that tetramers bound ankyrin with ten times greater affinity (K_D = 0.12 to 0.27 μ*M*).[134,135] Unfortunately, analysis by nondenaturing electrophoresis takes 40 to 48 h, undoubtedly leading to dissociation of ankyrin-spectrin complexes, and these studies differ from the initial immunoprecipitation assays which led to the conclusion that spectrin dimers and tetramers bind ankyrin with equal affinity in solution.[50] Finally, as spectrin dimers and tetramers bind to ankyrin with equal affinity *in situ*,[29] it would appear that at best the nondenaturing gel electrophoresis assay of spectrin-ankyrin interaction is yielding a result which is not representative of what occurs on the membrane.

Structural studies on human RBC ankyrin have thus far led to a provisional structural map of proteolytically derived domains.[136,137] Wallin et al.[136] and Weaver and Marchesi[137] have cleaved ankyrin into specific chemical and functional domains with trypsin and chymotrypsin. Based on peptide mapping of these major domains vs. overlapping fragments, the two laboratories have obtained nearly identical structural maps. The results of Wallin et al.[136] are summarized here:



The 11-kDa tryptic fragment of ankyrin is highly acidic (isoelectric point = 4.35) and its function has not yet been determined. The 83-kDa tryptic and chymotryptic domains are slightly basic (PI = 7.0 to 7.5), yield identical iodopeptide maps, and contain a binding site for band 3. Identification of the band 3 binding domain is based on the ability of the 83-kDa domain to inhibit ankyrin reassociation to ankyrin-depleted-inverted vesicles[136] and to bind to an affinity column to which the cytoplasmic domain of band 3 has been coupled.[134] The interaction of ankyrin with band 3 is discussed in greater detail in Section II.B.3.a. The 65-kDa tryptic domain

and 72-kDa chymotryptic domain of ankyrin are acidic (PI = 6.2 to 6.8), and contain the binding site for RBC spectrin.[134,136] The function of this domain has been demonstrated by the ability of the 65-kDa tryptic polypeptide to block the reassociation of spectrin with spectrin-depleted-inverted vesicles[136] and to bind to a spectrin affinity column.[134] It is now important to determine the complete sequence of RBC ankyrin so that its functional capabilities can be more completely understood at the molecular level.

RBC ankyrin is phosphorylated by cAMP-dependent and cAMP-independent protein kinases.[100,101] However, very little is known concerning the extent of phosphorylation *in vivo*, the localization of physiologically relevant phosphorylation sites, and the functional significance of ankyrin phosphorylation. At least two cAMP-dependent phosphorylation sites reside within the 65/72-kDa spectrin-binding domain of ankyrin.[126,134] This has led to interest in whether phosphorylation of ankyrin might regulate its interaction with spectrin. Lu et al.[135] published an estimate that each ankyrin contains three endogenous protein-bound phosphates as isolated. These authors further phosphorylated ankyrin with a purified cAMP-independent protein kinase in seven sites per ankyrin molecule. When tested in a nondenaturing gel electrophoresis assay, they found that phosphorylated ankyrin bound spectrin tetramer with an approximately fourfold reduction in affinity (K_D = 1.2 μM vs. 0.27 μM), while binding to spectrin dimer was unaffected. This unexpected result is difficult to interpret because of (1) the flaws in the nondenaturing gel electrophoresis assay, (2) the lack of knowledge concerning whether the seven phosphorylation sites are physiologically relevant, and (3) where they are located within the ankyrin domain map. Finally, if we take these measurements at face value, one must still recognize that a shift in K_D from 0.27 to 1.2 μM will have little effect on the membrane surface where the concentration of ankyrin is >200 mM. Avian and mammalian RBC ankyrin are also fatty acid acylated, but the functional significance of this posttranslational modification has not yet been studied.[138]

The spectrin-ankyrin interaction is normal in most subjects with HS,[61,62,127] but a kindred with atypical HE demonstrated a defective binding of spectrin to spectrin/actin-depleted-inverted vesicles and to the purified 72-kDa fragment of ankyrin.[139] Therefore, a defective spectrin-ankyrin interaction can lead to a weakened and misshapen red cell. In addition, spectrin/actin-depleted-inverted vesicles from sickle cells of symptomatic patients bind ~50% less spectrin than do control vesicles.[129] Interestingly, ankyrin isolated from these sickle cells bound spectrin normally, leading to the possibility that either some other protein involved in the interaction was altered or, more likely, that the authors were isolating a normal subpopulation of sickle cell ankyrin.

b. Protein 4.1

Protein 4.1 remains firmly attached to the bilayer of inside-out vesicles prepared by low ionic strength extraction of ghost membranes or NaOH extraction of membranes at pHs of 11 to 11.5.[140,141] These are conditions which completely remove spectrin and actin. Since 4.1 is a component of the ternary spectrin-actin-4.1 complex discussed earlier, it is also able to play the role of a linking protein which helps to maintain the tight attachment of the spectrin skeleton to the membrane. Evidence for the specificity and tightness of the 4.1-membrane interaction was provided by Shiffer and Goodman,[95] who determined that the interaction was saturable (K_D of 42 to 50 nM), pH and ionic strength dependent, and had a maximal binding capacity of 60 to 70 μg 4.1 per milligram ghost protein. This latter value corresponds to the physiological amount of 4.1 found on the membrane, which is ~200,000 copies per cell.[24] Values of the same order of magnitude for K_D of a high-affinity binding site were reported by Pasternack et al.,[142] although they also proposed a low-affinity binding site with a K_D of $5 \times 10^{-7} M$ and a capacity to bind far more than the physiological amounts of 4.1. Both isomorphs of 4.1, a and b, bind to inside-out stripped vesicles in the same ratio as they are found on the ghost membrane.[95] Whether 4.1 a and 4.1b subserve different functions in their role as linking proteins has not been established, nor has a role been defined for other 4.1 family members that appear to be consistently present on

the ghost membrane,[87,89] e.g., the 87/85- and 67-kDa members. Evidence that protein 4.1 links spectrin and actin to the membrane by also binding to glycophorin A or C is discussed in Section II.B.3.b.

The importance of 4.1 in maintaining normal RBC shape can be seen by inspection of RBCs from certain individuals who exhibit a deficiency of the protein. Red cells from individuals with no or reduced amounts of 4.1 are elliptical,[143-145] as are cells of certain individuals with 4.1 of variant molecular weights.[146,147] Takakuwa et al.[148] reported that the unstable membranes of elliptocytes from a 4.1-deficient individual could be stabilized by exchange hemolysis with 4.1. Membranes reconstituted in such a fashion were more stable to mechanical stresses, but since the amount of 4.1 trapped within the reconstituted ghosts was three to five times higher than normal, the effect of the nonspecifically loosely bound 4.1 cannot be defined. In these studies, however, a fraction of the incorporated 4.1 was tightly associated with the membrane, and in view of the fact that excess 4.1 added to normal RBCs did not change membrane stability, the results indicate that exogenous 4.1 did restore some of the membrane properties of 4.1-deficient RBCs. Recently, the gene for protein 4.1 has been localized to chromosome 1,[149] and in one family with HE and total 4.1 deficiency, a DNA rearrangement leading to altered mRNA splicing was identified.[149] Inversion of a short DNA segment, or deletion of a short sequence, was thought to be involved in the mutation. This region of chromosome 1 is also very close to the locus for the Rh factor, and since isolated cases of elliptocytosis have been genetically linked to a lack of Rh antigens[150,152] and since the Rh polypeptide is linked to the membrane skeleton,[151] it is possible that the 4.1 and Rh loci are linked. In view of this evidence, the conclusion that protein 4.1 plays a role in maintaining erythrocyte shape and membrane stability is convincing.

3. Integral Membrane Protein Attachment Sites

In Sections II.B.2.a and b, we discussed the role of ankyrin and protein 4.1 as essential linkers of the spectrin/actin skeleton to the bilayer. In this section, we discuss the proteins which are embedded within the bilayer and which serve as the primary sites of attachment for ankyrin and protein 4.1.

a. Band 3

Band 3 is a multifunctional integral membrane glycoprotein, which constitutes approximately 25% of the total erythrocyte protein and is present in about 1×10^6 copies per cell (Table 1).[24,85] The protein has a monomer molecular weight of 90,000 to 100,000 Da. A single branched asparagine-linked carbohydrate chain of ~30 sugar residues representing 5500 Da of the molecular mass is located on an extracellular domain of band 3.[153-156] In the human RBC membrane, band 3 appears to be present as dimers and tetramers,[157-161] although the relative percentage of each oligomeric form and factors which regulate their interconversion are unknown. The many important functions of band 3 include

1. Serving as an anion antiporter which mediates the one-for-one obligatory and electroneutral exchange of chloride and bicarbonate across the membrane, thereby increasing the capacity of the blood to transport CO_2 from the tissues to the lungs.[162-166]
2. Facilitating the osmotically driven movement of water across the RBC membrane.[167-169]
3. Carrying the antigenic determinants for the A, B, and O blood groups[170,171] and possibly Rh[172] and Kell antigens.[173]
4. A subpopulation of band 3 is converted during red cell aging to the senescent cell antigen which is recognized by autologous antibodies, an event which triggers removal of the senescent red cell from the circulation by macrophages.[174,175]
5. The cytoplasmic segment of band 3 serves as the binding site for enzymes (glyceraldehyde-3 phosphate dehydrogenase, aldolase, phosphofructokinase, catalase), hemoglobin and hemichromes, membrane protein 4.2, and ankyrin.[176-191]

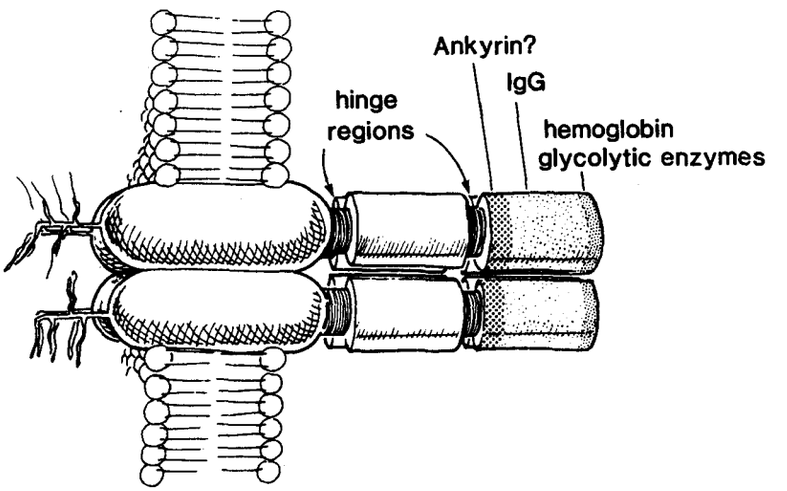


FIGURE 4. The cytoplasmic domain of band 3. We present a structural model of the 43-kDa N-terminal cytoplasmic domain of band 3. Also included are suggested binding sites for glycolytic enzymes, IgG, hemoglobin, and ankyrin. As discussed in the text, the position of the ankyrin-binding site is highly speculative.

Mild proteolytic digestion of sealed inside-out erythrocyte vesicles which have been depleted of peripheral membrane proteins, cleaves band 3 into a 50- to 55-kDa extracellular and membrane-spanning domain which retains anion transport function,[192-194] and a 43-kDa N-terminal cytoplasmic domain which retains the capacity to bind ankyrin and the other proteins listed above. An excellent recent review has appeared which stresses the structure and function of the cytoplasmic 43-kDa domain of band 3.[195] We focus here primarily on the interaction of ankyrin with the cytoplasmic domain of band 3.

The 43-kDa chymotryptic or tryptic cytoplasmic fragment of band 3 is released in water-soluble form from spectrin-actin-depleted vesicles which have been further depleted of ankyrin, band 4.1, and band 6 by acetic acid stripping.[196,197] Based on hydrodynamic studies, the 43-kDa domain has a Stokes radius = 5.3 nm, $S_{20,w} = 4.1$, and a calculated molecular weight = 95 kDa, based on a partial specific volume of 0.74 cm^3/g.[197] Therefore, the 43-kDa domain is isolated as a homodimer of two 43-kDa polypeptides. The calculated frictional ratio (f/fo = 1.6) is consistent with an axial ratio of 10.[197] Low[195] has recently calculated that, based upon the simplifying assumption that the dimer is roughly cylindrical in shape, it would be about 2.5 nm in diameter and 25 nm in length. Interestingly, deep-etched electron micrographs of the cytoplasmic surface of erythrocyte membranes stripped of peripheral proteins have demonstrated elongated finger-like protrusions which have been shown to represent the cytoplasmic domain of band 3.[157] Based upon hydrodynamic and fluorescence energy transfer studies, Low et al.[198] have found that the 43-kDa domain undergoes a pH-dependent change which has been suggested to involve the pivoting of two subdomains at a central flexible hinge region. A model of the 43-kDa domain which indicates suggested functional sites is presented in Figure 4.

Kaul et al.[199] sequenced a 201 amino acid 23-kDa S-cyanylation fragment representing the N-terminal region of band 3. The N-terminus of the 23-kDa fragment has a stretch of 75 amino acids which are highly acidic. Of the first 37 amino acids, 18 were acidic and the rest were neutral.[199] This highly acidic N-terminal segment of the 43-kDa domain contains the binding sites for the glycolytic enzymes, hemoglobin, and hemichromes (Figure 4). Kopito and Lodish[200] have reported the complete nucleotide sequence and predicted amino acid sequence of cDNA encoding mouse erythrocyte band 3. The predicted polypeptide had a molecular weight of 103

kDa, with a 420 residue negatively charged N-terminal domain, a 450 residue amphipathic central domain, and a highly acidic (ll Glu + Asp) 32 residue C-terminal segment. Based on sequence analysis, band 3 probably possesses 12 membrane-spanning domains, 5 of which are linearly hydrophobic and 7 are amphipathic helical structures.[200] Recently, the nucleotide sequence and predicted amino acid sequence of a cDNA-encoding K562 cell nonerythroid band 3 has been reported by Demuth et al.[201] A comparison of this sequence, with the sequence of mouse and human RBC band 3 indicated a high degree of sequence homology in the membrane-spanning domains, with little homology in the N-terminal cytoplasmic domain, except for amino acids 157 to 177 which demonstrated 75% homology, with residues 167 to 177 showing 100% homology between human RBC band 3 and human K562 band 3. It has been suggested that this conserved sequence (157 to 177) might represent the ankyrin-binding site,[201] while residues 125 to 138 represent the IgG-binding site in mouse RBC band 3,[200,202] and residues 186 to 204 could represent the hinge region[178] for the cytoplasmic domain of mouse RBC band 3.[200] Band 3 is phosphorylated *in vivo* by cAMP-independent protein kinases, with an average of one phosphate per band 3 monomer.[203,204] There are at least three phosphorylation sites within the 43-kDa domain. The most extensively phosphorylated site is tyrosine-8, which is phosphorylated by a tyrosine kinase that is associated to the membrane skeleton through its attachment to band 3.[205,206] Secondarily, serine and threonine residues which are located closer to the membrane, within 11 kDa of the C-terminus of the cytoplasmic domain, are phosphorylated by a cAMP-independent membrane-associated protein kinase.[203,204] Phosphorylation of two tyrosine residues within the acidic N-terminal region of the 43-kDa domain by an exogenous tyrosine kinase blocked the binding of aldolase, glyceraldehyde 3-phosphate dehydrogenase, phosphofructokinase, and hemoglobin to band 3.[207]

The initial suggestion that band 3 might serve as the attachment site for ankyrin was based upon studies by Bennett and Stenbuck,[191] indicating that antibody against the spectrin-binding domain of ankyrin immunoprecipitated ankyrin and band 3 in a 1:1 mol/mol complex from Triton X-100-solubilized spectrin-depleted-inverted vesicles. While the conclusion of this paper was limited by the possibility of nonspecific association of membrane proteins after detergent solubilization and the coprecipitation of band 4.2 with band 3 and ankyrin, subsequent studies demonstrated conclusively that it was correct. Bennett and Stenbuck[196] demonstrated that antibodies against the 43-kDa domain of band 3 could precipitate ankyrin and band 4.2 along with band 3 from Triton X-100-solubilized-inverted vesicles. More importantly, they showed that the purified 43-kDa domain of band 3 bound ankyrin in solution with a $K_D = 8$ n*M* in an ~1:1 mol/mol complex. ^{125}I-ankyrin was demonstrated to reassociate with ankyrin-depleted-inverted vesicles (24°C) at high-affinity (K_D = 4.6 n*M*) and low-affinity (K_D = 140 n*M*) sites with saturation at 65 to 75 μg ankyrin per milligram ghost protein. The binding to high-affinity sites was 90% inhibited and low-affinity binding 60% inhibited by preincubation of the inverted vesicles with anti-43-kDa IgG. Binding was eliminated by mild chymotrypsin digestion of inverted vesicles, which releases the 43-kDa domain of band 3. Furthermore, the purified 43-kDa fragment could compete with vesicles for the binding of ^{125}I-ankyrin with a K_I value = 660 n*M*.[196] These findings, which clearly demonstrated that band 3 serves as the binding site for ankyrin on the erythrocyte membrane, were confirmed by others.[208] Hargreaves et al.,[208] performing similar binding studies of ^{125}I-ankyrin to ankyrin-depleted-inverted vesicles (except at 0°C and physiological ionic strength), showed binding to a single class of high-affinity sites with a K_D value = 50 to 80 n*M* and a stoichiometry of one ankyrin per eight to ten band 3 molecules. Once again proteolytic digestion of the vesicles eliminated binding of ^{125}I-ankyrin, and the 43-kDa fragment inhibited reassociation to undigested ankyrin-depleted vesicles with a K_I = 500 n*M*. Glyceraldehyde 3-phosphate dehydrogenase (band 6) had no affect on binding up to a concentration of ~4 μ*M*. Vesicles depleted of band 4.2 as well as ankyrin rebound ^{125}I-ankyrin with equal affinity and capacity to ankyrin-depleted vesicles. These studies each indicated a K_I value for the 43-kDa fragment's inhibition of ^{125}I-ankyrin binding to ankyrin-

depleted-inverted vesicles (500 to 660 n*M*) which was far greater than the K_D value of membrane reassociation (4.6 to 140 n*M*). This suggests that some additional protein may be involved in the reassociation. Two pieces of information may be relevant to this point. Based upon the ability of antibodies against glycophorin A to suppress the rotational diffusion of band 3, Nigg et al.[209] concluded that band 3 and glycophorin A are associated in the membrane. Hsu and Morrison[210] found that the anion transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)′, which binds to an extracellular domain of band 3, causes a decrease in the extractability of ankyrin and spectrin upon 0.1 *N* NaOH incubation of erythrocyte membranes. Interestingly, trypsin digestion of intact DIDS-pretreated erythrocytes released the tightened association of ankyrin and spectrin to band 3. Since trypsin does not cleave band 3 from the outside of intact erythrocytes, this result suggests that some other protein which is exposed on the outer surface of the erythrocyte (perhaps glycophorin A) may modulate the ankyrin-band 3 interaction.

An interesting unanswered question is why are only 10 to 15% of the band 3 molecules involved in binding to ankyrin? While the simplest explanation, that the content of ankyrin in the red cell (100,000 copies) is one tenth the concentration of band 3 (1×10^6 copies), may be part of the answer, it does not explain why the maximal binding capacity of ankyrin-depleted-inverted vesicles is one ankyrin bound per eight to ten band 3 monomers.[196,208] This result suggests that either ankyrin can bind to an active subpopulation of band 3 molecules or, alternatively, can bind only to a specific oligomeric form of band 3. The former possibility does not appear to be true because (1) two-dimensional tryptic and chymotryptic peptide maps of ankyrin-associated band 3 and unbound band 3 are identical;[211] (2) one-dimensional peptide maps of human, mouse, and pig band 3, which is Triton-soluble or Triton-insoluble because of association with the skeleton through ankyrin, are identical;[87] and (3) the 43-kDa domain isolated from ankyrin-bound or free band 3 have equal ability to block the binding of ^{125}I-ankyrin to ankyrin-depleted-inverted vesicles.[208] This leaves the possibility that binding of ankyrin is based on the oligomeric structure of band 3. As mentioned earlier, the ratio of band 3 oligomeric structures within the membrane and what regulates their interconversion are subjects for future research.

The exact location of the ankyrin-binding site along the 43-kDa cytoplasmic domain of band 3 is not known. It has been suggested that the ankyrin-binding site might reside between the hinge region and the IgG-binding site,[195,198] based on the fact that glyceraldehyde-3-phosphate dehydrogenase did not block binding of ^{125}I-ankyrin to ankyrin-depleted-inverted vesicles,[208] while anti-43-kDa IgG could block binding.[196] Furthermore, a 21 amino acid segment of the 43-kDa domain (residues 157 to 177) has been suggested as a potential ankyrin-binding site because it is highly conserved in mammalian erythroid and nonerythroid band 3 and is found between the hinge region and IgG-binding region.[201] While this suggestion can be easily tested, the proposal that the ankyrin-binding site resides between the hinge and IgG-binding regions was based on two problematic assumptions. The inability of dilute solutions of glyceraldehyde-6-phosphate dehydrogenase (band 6) to block binding of ^{125}I-ankyrin to ankyrin-depleted vesicles[208] was most probably due to the inability of this glycolytic enzyme to bind to the 43-kDa domain of band 3 at physiological ionic strength and pH[212] and therefore should not be taken as proof of ankyrin not binding to the acidic N-terminus of this domain. Secondly, the ability of intact IgG against the 43-kDa domain to block binding of ^{125}I-ankyrin to ankyrin-depleted vesicles[196] cannot be utilized to precisely localize the ankyrin attachment site because the geometry of an IgG molecule indicates that the linear dimension of the two antigen-binding Fab fragments is 14.2 nm,[213] while the total length of the 43-kDa domain has been estimated as 25.0 nm.[195] Based on these geometric considerations, an IgG molecule bound to a region between the acidic N-terminus and the hinge by one of its Fab regions could sterically block binding of another protein to the 43-kDa domain almost independent of its site of binding. For these reasons, in Figure 4 we demonstrate Low's suggested ankyrin-binding site,[195] but place a question mark next to it.

Soong et al.,[214] utilizing a purified cAMP-independent protein kinase to phosphorylate the 43-kDa domain of band 3, found no effect upon its ability to bind ankyrin. Phosphorylation by the exogenous kinase placed ~1 mol phosphate per mole 43-kDa domain, with phosphorylation of serine and threonine residues and to a lesser extent tyrosine. The interpretation of these data is difficult because the phosphorylation sites were not precisely localized, and it is not at all clear that they are physiologically relevant sites. Alternatively, these authors found that phosphorylation of ankyrin with this cAMP-independent kinase (7 mol phosphate per mole ankyrin) altered the stoichiometry of the ankyrin-band 3 interaction as measured by nondenaturing gel electrophoresis. The interpretation of these results suffers from the same problems, in addition to the fact that the phosphorylation sites on ankyrin appear to reside within the 72-kDa spectrin-binding domain, not the 83-kDa band 3-binding domain,[126,134] and problems with the nondenaturing gels electrophoresis technique have already been described (Section II.B.2.a). The question of whether the ankyrin-band 3 interaction is regulated by phosphorylation is unresolved and will remain so until phosphorylation into sites which are carefully localized and physiologically relevant are tested for their affect upon this interaction.

As mentioned in Section II.B.2.b, binding of ^{125}I-protein 4.1 to 4.1-depleted-inverted vesicles has indicated a high-affinity interaction (K_D = 20 to 50 n*M*) which saturates at the physiological concentration of membrane-associated protein 4.1,[95,142] and a low-affinity interaction (K_D = 500 to 675 n*M*)[142,215,216] which saturates at levels far above the physiological concentration of protein 4.1. Because the physiological content of protein 4.1 can be reassociated with the membrane through the high-affinity binding sites (discussed further in Section II.B.3.b) and since saturation of low-affinity sites reaches values as high as 700 μg 4.1 per milligram vesicle protein or 350 μg 4.1 per milligram ghost protein,[216] which is five times the physiological concentration of membrane-associated protein 4.1, we have considered the low-affinity binding to be non-physiological and not worthy of extensive consideration. However, Pasternack et al.[142] studied the low-affinity interaction and found, that at high concentrations of ^{32}P-protein 4.1, antibodies against glycophorin A could reduce binding by 30% to 4.1-depleted-inverted vesicles, while antibody against the 43-kDa band 3 cytoplasmic domain reduced binding by 65%. In addition, these authors found that the 43-kDa fragment could displace 65% of the ^{32}P-4.1 binding with a K_I = 4.65 μ*M*. From these studies, they concluded that band 3 represents the low-affinity binding site for protein 4.1 on the erythrocyte membrane. Interestingly, the 43-kDa fragment was found to bind to protein 4.1 in solution and to displace bound spectrin.[142] If this were operational *in situ*, then those protein 4.1 molecules bound to band 3 could not serve to link the skeleton to the membrane. The site of protein 4.1 binding on the 43-kDa fragment is unknown, and competition studies with ankyrin binding have not been performed.

Band 3 is restricted in its lateral mobility within the membrane by its interactions with and entrapment by the spectrin membrane skeleton.[1] The diffusion constant of band 3 in artificial lipid vesicles is 1.6×10^{-8} cm^2/s at 30°C, but is ~3000 times slower in the erythrocyte membrane ($D = 4$ to 6×10^{-11} cm^2/s).[217] Sheetz et al.[218] have reported a diffusion constant of 2.5×10^{-9} cm^2/s for band 3 within the RBC membranes of spectrin-deficient mutant mice, while the diffusion constant in normal mouse RBC membranes was 4.5×10^{-11} cm^2/s at 24°C. Furthermore, incubations of human RBC membranes at ionic strengths which enhance spectrin dimer to tetramer formation slow diffusion ($D = 5 \times 10^{-12}$ cm^2/s).[219] Therefore, the lateral mobility of band 3 within the membrane is restrained both by its interaction with spectrin through ankyrin and by its entrapment within a network of spectrin tetramers linked to actin protofilaments.

b. Glycophorins A and C

Attempts to identify membrane-binding sites for protein 4.1 have been made by various methods and have yielded varying results. Analysis of the membrane skeleton prepared by Triton X-100 extraction of ghosts[20,141,220] showed that nearly all of the glycophorin C (β in the widely used nomenclature of Anstee et al.[221]) stayed with the skeleton in normal RBCs, but in

a patient with 4.1 deficiency, β was missing from the skeleton. Furthermore, glycophorin β could be extracted by Triton, but only after 4.1 was stripped from the membrane. Glycophorin A (α) was primarily extracted by the detergent, with only a small proportion of the monomer and none of the dimer remaining with the skeleton.[20,141] This led to the conclusion that β was the binding site for 4.1. At the time, the difficulty with this suggestion was that there are about 50,000 copies of β (actually β plus a closely related glycoprotein β_1, as per Anstee et al.[221]) but 4 times as many copies of 4.1 per cell. Elliott and Ralston[222] extracted inside-out vesicles with Tween 20 in glycine-NaOH buffer, pH 9.8, and analyzed the extract. They found that 4.1 and sialoglycoproteins copurified by gel filtration, ion exchange, and affinity chromotography. These authors also confirmed the relationship of α and β to the shell as discussed previously.

Several investigators have attempted to determine the identity of the 4.1-membrane-binding site by investigating 4.1 binding to inside-out vesicles or to artificial phospholipid vesicles. In the studies of Shiffer and Goodman,[95] chymotryptic and tryptic digestion of 4.1-stripped vesicles indicated that removal of the cytoplasmic portion of band 3 did not inhibit high-affinity 4.1 binding, but digestion of vesicles with papain (which removed 67% of glycophorin α dimer) decreased 4.1 binding by 61%. These authors concluded that the major high-affinity binding site was protein, but suggested caution in concluding that the binding site resided in glycophorin α because most of α does not remain with the skeleton and because papain digestion of inverted vesicles also proteolyzes other proteins.

Binding of 4.1 to artificial phosphatidylserine-containing membranes and inside-out vesicles was studied by Sato and Ohnishi.[223] They reported that 4.1 bound to phosphatidylserine-containing liposomes with higher affinity than to other phospholipid vesicles and that binding of 4.1 to inside-out red cell membranes was inhibited after treatment of the vesicles with phosphatidylserine decarboxylase. However, the authors state that the enzyme preparation contained significant amounts of residual detergent, and due to the detergent, certain concentrations of enzyme caused loss of 4.1 from the vesicles. Normally, 4.1 remains with the shell after detergent (Triton X-100) treatment, but in the control experiment from this study, 4.1 was lost. This would cast doubt upon the validity of the conclusion that less 4.1 bound to vesicles that had less phosphatidylserine.

Two reports by Anderson and co-workers[224,225] presented evidence that glycophorins were the binding site for 4.1. In these studies, liposomes made from phosphatidylcholine, incorporating mixed and desialated glycophorins, bound more 4.1 than vesicles without the glycoproteins. When anti-glycophorin α antibodies were used to block the cytoplasmic tail of α, 70 to 80% of the 4.1 binding was blocked. If vesicles were made of purified α, the antibodies blocked over 90% of the binding. Antibodies preincubated with stripped, inside-out vesicles of RBC membranes blocked only 60% of the 4.1 binding. From these data, the authors concluded that glycophorin α provides 60% of the 4.1 binding sites, and β and δ provided the remaining sites. A control which is missing from these studies was to test 4.1 binding to liposomes which had incorporated other integral proteins (since 4.1 is a very sticky molecule) and to use antibodies against other membrane proteins such as band 3. Controls of this type are necessary because glycophorin α may be bound to band 3 *in vivo*[209] and band 3 has also been proposed as a binding site[142] as was discussed in Section II.B.3.a. Regulation of 4.1-glycophorin binding by polyphosphoinositides has also been proposed.[225] The ability of micellar glycophorin to bind 4.1 was dependent on the presence of the inositides, with phosphatidylinositol 4,5-bisphosphate bestowing a higher apparent affinity for 4.1 than phosphatidylinositol 4-phosphate. Other phospholipids were ineffective. Liposomes containing glycophorin and phosphatidylinositol 4,5-biphosphate were able to compete with inside-out RBC membrane vesicles for 4.1 binding, but those containing glycophorin and phosphatidylserine were not, suggesting that the affinity of phosphatidylserine for 4.1[223] is much lower.

It has become very apparent in recent studies, that all glycophorins are not alike, and that use of a mixture of glycophorins will not lead to identification of specific binding sites for 4.1. The

complete amino acid sequence of glycophorin α, β, and δ (A, C, and B) have been determined.[226-230] Glycophorins α and δ are identical for the first 26 amino acids, but there is less homology between α and β. Blood group antigens, which are carried on the carbohydrate portions of these molecules differ entirely. Monoclonal antibodies have been developed, and used successfully to distinguish the glycophorins and to allow their quantitation in the membrane.[231-233] By using iodinated monoclonals, estimates of the number of copies of various glycoproteins include: α, 600,000; β and β_1, 50,000; and δ, 80,000. Approximate molecular weights determined from the migration of these molecules on 10% Laemmli gels are α_2, 83,000; β_2, 75,000 to 83,000; δ_2, 50,000; α, 43,000; β, 39,000; γ, 30,000; and δ, 25,000. Use of the monoclonals has also led to the identification of a large number of sialoglycoprotein variants, to the discovery of cross-hybridization, and even to an instance of double crossover.[221,232-240] To date, the crossovers that have been identified are α-δ and δ-α hybrids, α-δ-α double hybrids, and β-γ hybrids. An interesting finding in regard to the variants and hybrids is that many of them have been associated with variations in red cell shape and membrane stability. Shape changes have also been correlated with the absence of normal sialoglycoproteins. In two unrelated patients with elliptocytosis, the membranes were found to lack β, β_1, and γ.[241] Cells of individuals of the Ge(–) phenotype also lack β and γ, but in addition contain variant molecules of 30,000 to 34,500 and 32,500 to 36,500 apparent mol wt.[242-244] These cells have a normal shape and normal amounts of 4.1, which suggest that the variant glycoproteins still contain the 4.1-binding site and provide a normal skeletal structure. Sondag et al.[244] studied a family which was classified as a 4.1 (–) phenotype, where 4.1 deficiency was considered the primary defect. The membranes of the homozygous condition had a 70% reduction in β and γ, and the heterozygote had moderately reduced β. It was suggested that 4.1 controlled the amount of the glycoproteins in the membrane. However, there was no evidence to show that in fact the glycoprotein deficiency was not the primary defect. Cells from these patients were elliptocytic. In another family with homozygous and heterozygous 4.1(–) HE, β was absent or reduced and γ was decreased.[245] Erythrocytes of the En(a–) type lack α but the cells have a normal shape and 4.1 content,[246,247] which indicates that α cannot be the sole or even most important binding site. Recent studies[248] using the ektacytometer to assess membrane stability and deformability have shown that membranes that lack either α or δ are normal, while membranes which lack β and γ had a 50% decrease in mechanical stability and a 40% decrease in deformability. These cells are also elliptocytic, but have a normal 4.1 content.

Recent studies by Whitfield et al.[249] have attempted to use heterobifunctional cross-linkers to determine the binding sites for 4.1. The cross-linker, ^{125}I-labeled Denny Jaffee reagent, was covalently linked to purified 4.1, and the labeled 4.1, at concentrations near the K_D, was allowed to bind to stripped inside-out vesicles. Following photolysis to allow covalent attachment of the labeled end of the reagent to the sites nearest to 4.1, the cross-linker was cleaved and 4.1 restripped from the vesicles. Location of the label was then assessed on autoradiographs of solubilized vesicles run on SDS-PAGE. Results demonstrated that transfer of label from native 4.1 but not denatured 4.1 occurred to molecules migrating with molecular weights of 52 and 34 kDa, and bands labeling equally well with native or denatured 4.1 migrated at 80 and 71 kDa. It appears that the lower molecular weight bands correspond to glycophorins β or β_1 and that these are the specific binding sites, while the higher molecular weight bands represent nonspecific binding. Further studies were done to specifically immunoprecipitate tritiated borohydride-labeled membrane sialoglycoproteins with 4.1 antibodies after 4.1 binding to stripped vesicles. Data from these experiments showed specific immunoprecipitation of three sharp bands in the 83- to 79-kDa range, which may be a small amount of α_2 and β_2, two sharp bands comigrating with β and α monomers, and one band which comigrates with γ. The quantity of the β and γ bands that immunoprecipitated increased with the amount of 4.1 bound. Similar findings from both of these independent approaches lend support to the hypothesis that the high affinity binding sites for 4.1 are glycophorins β and γ and that a small amount of binding occurs to the α dimer, but the latter appears to be nonspecific.

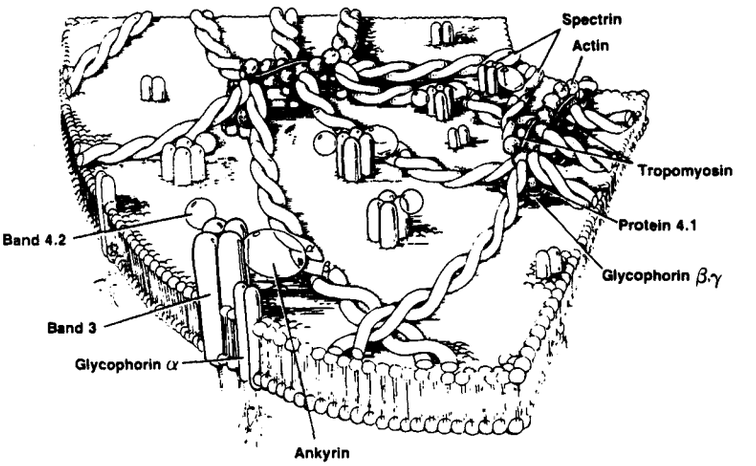


FIGURE 5. Model of the human erythrocyte membrane skeleton. An updated model of the protein interactions in the human erythrocyte membrane skeleton. This model is consistent with all data discussed in the text, but does not include protein 4.9 because of a lack of detailed knowledge concerning the function of this protein.

The predominant evidence discussed here suggests that glycophorins β and γ are the primary high affinity binding sites for 4.1. Relationships between 4.1, glycophorins, and red cell shape are less clear. It seems that elliptocytosis is the only shape abnormality so far associated with lack of 4.1 or glycophorin β. Lack of α has not been associated with a lack of 4.1 or with specific hemolytic anemias. A deficiency of β and γ is associated with elliptocytosis in some cases but not others (e.g., the Ge[–]), although it may be that if a hybrid glycophorin which retains the binding site is present then no cell abnormality occurs. A distinct survival advantage would be gained by having multiple binding proteins for a major membrane skeletal protein, each controlled by separate genes. In terms of the overall stoichiometry of 4.1-membrane binding, it would on first thought seem to require 200,000 binding sites, but the total of glycophorins β, β_1, γ, and perhaps hybrids of these as yet unidentified, would not exceed 100,000. Numbers of copies of the minor glycophorins are not certain since they are hard to quantify and monoclonal antibodies to all of them are not yet available. However, assuming that there is an important functional reason for the isomorphs of 4.1, a and b, a model could be envisioned where one isomorph binds to the membrane-binding site and the other one to the first isomorph and to spectrin. This possibility cannot be ruled out on the basis of any of the studies of 4.1 binding done to date.

C. Synthesis, Assembly, and Turnover

In the previous sections, we discussed the studies leading to the model of the spectrin membrane skeleton presented in Figure 5. Spectrin $(\alpha\beta)_2$ heterotetramers are cross-linked into a two-dimensional network through the interaction of short actin protofilaments (~13 actin monomers) and protein 4.1 with their ends. The actin protofilaments and protein 4.1 both probably associate with both subunits of spectrin. Tropomyosin lies within the grooves of the actin protofilament, strengthening the f-actin and protecting it from erosion of actin monomers. The precise physiological role of protein 4.9, an actin-binding protein, remains to be determined. This two-dimensional spectrin/actin skeleton is attached to the membrane through interaction of a single ankyrin molecule with a β subunit of spectrin ~20 nm from the center of the $(\alpha\beta)_2$

tetramer and through interaction of protein 4.1 at the termini of the spectrin tetramer. Ankyrin is, in turn, associated with the cytoplasmic N-terminal segment of band 3, although the favored oligomeric state of band 3 for ankyrin binding has not been determined. Protein 4.1 is probably associated to the membrane through its interaction with glycophorins β and γ, although this is a subject of current debate.

Most of the studies leading to the model described here have been performed on human erythrocyte membranes, while studies on membrane skeleton synthesis, assembly, and turnover are more easily performed on erythroid precursor cells from small mammals and birds. Mouse, rat, pig, and chicken erythrocytes contain similar, but not identical, membrane skeletal components to those contained in human erythrocytes.[87,250] While considering the studies dealing with membrane skeletal morphogenesis which are discussed below, it is important to keep in mind, that functional similarities between murine, avian, and human erythrocyte skeletal proteins are in most cases only presumed rather than established.

Murine band 3 is synthesized on membrane-bound polysomes, and is cotranslationally inserted into the membrane of the endoplasmic reticulum in its proper orientation, where it acquires its core oligossaccharide.[251-253] There does not appear to be an N-terminal signal sequence, but instead a sequence near the middle of the protein probably directs its insertion into the membrane.[252,253] Band 3 passes from the endoplasmic reticulum to the Golgi, where its oligossaccharide is completed, and reaches the cell surface about 20 to 30 min after its synthesis.[251-253] Unlike mammalian band 3, avian band 3 is composed of two polypeptides with an apparent molecular weight of 100 and 105 kDa.[254] The two avian band 3 polypeptides are closely related in sequence and share antigenic sites,[255] yet their oligomeric state in the membrane and individual capacity to bind ankyrin has not been determined. Weise and Chan[250] demonstrated that the synthesis of band 3 (100 and 105 kDa) in primitive chicken embryo erythroid cells is greater than in definitive erythroid cells, but that all major erythrocyte membrane proteins are synthesized simultaneously in differentiating erythroid cells.

Most work on the synthesis, assembly, and turnover of the skeletal proteins spectrin, 4.1, and ankyrin has been performed in embryonic avian erythroid cells. In avian embryonic erythrocytes, the α subunit of spectrin, which is synthesized on polysomes which are associated with the cytoskeleton,[256] is synthesized in a threefold molar excess to the β subunit of spectrin.[257-259] The greater synthesis of α spectrin is due to the α-spectrin mRNA being 2.5 to 3 times more prevalent than β spectrin mRNA in avian embryonic erythrocytes.[257,258] The spectrin subunits rapidly assemble on the membrane in a 1:1 mol/mol stoichiometry, with the large soluble pool of remaining α spectrin and the small soluble pool of β spectrin rapidly turning over.[257,258] Unassembled α spectrin is degraded ($t_{1/2}$ = 2 h) by a system with the characteristics of an ATP-dependent membrane-bound lysosomal pathway, while β spectrin is degraded extremely rapidly ($t_{1/2}$ = 15 min) by a soluble ATP-independent cytoplasmic system.[260] There is only a small soluble pool of ankyrin in avian embryonic erythroid cells, which is rapidly incorporated into the cytoskeletal fraction. Cytoskeletal spectrin and ankyrin, once assembled, does not turnover, while soluble ankyrin is catabolized with a $t_{1/2}$ = 1 h. Moon and Lazarides,[259] using a chicken embryo erythroid lysate system, tested the ability of newly synthesized spectrin subunits to bind to spectrin-depleted-inverted erythrocyte vesicles. A total of 95% of the newly synthesized β spectrin and equal amounts of α spectrin bound to the membranes, with excess α spectrin remaining unbound. Spectrin-depleted vesicles could be added after synthesis was complete and the vesicles still bound the newly synthesized spectrin, suggesting that assembly of spectrin on the membrane is posttranslational.

The results described here led to the "receptor mediated assembly and stabilization hypothesis", which suggests that spectrin α and β subunits and ankyrin are all synthesized rapidly and concurrently and are assembled posttranslationally into stoichiometric complexes based on the amount of each protein binding to its receptor, rather than the amount of each protein synthesized. In other words, ankyrin will bind to the number of newly synthesized band 3

molecules in binding competent oligomeric form, with remaining unbound ankyrin rapidly catabolized. β-Spectrin assembly would be linked by the number of ankyrin molecules bound and, in turn, α spectrin assembly would be limited by the number of assembled β spectrin molecules.[261] While this hypothesis seems intuitively logical, it is highly speculative because the functional interactions of skeletal proteins derived from avian embryonic erythrocytes have not been established, and the model simplistically does not consider the influence of other skeletal proteins (4.1, tropomyosin, actin, and glycophorins β and γ) upon assembly. Recently, it has been demonstrated that 96% of newly synthesized avian RBC protein 4.1 becomes assembled within 5 min of synthesis either co- or posttranslationally.[262] It appears that protein 4.1 assembly may be determined largely by the availability of protein 4.1 polypeptides synthesized, rather than by the number of 4.1-binding sites available. Interestingly, a recent article by Woods and Lazarides[263] seems to present an alternative explanation for the regulation of erythrocyte membrane skeletal assembly and turnover. It appears that, after synthesis of avian α and β spectrin monomers, they rapidly assume one of two configurations: (1) a soluble αβ heterodimer which can associate with the membrane, or (2) a homooligomer (β_4 or α_2) which is rapidly degraded. The β_4 oligomer binds to inside-out spectrin-depleted vesicles with substantially lower affinity than the αβ heterodimer, while the α_2 oligomer does not bind. Therefore, contrary to this laboratory's previous model,[261] these results would suggest that the assembly of spectrin subunits is limited by the formation of either binding competent or incompetent spectrin oligomers.[263] It should be obvious that much more work is needed to understand the regulation of spectrin membrane skeleton assembly in avian erythroid cells. Furthermore, we would suggest that the most fruitful avenue for future research would be on the synthesis, assembly, and turnover of the spectrin membrane skeleton in mammalian precursor erythroid cells, where the functional capacity of these proteins has been firmly established.

III. NONERYTHROID SPECTRIN

A. Discovery

In Section II, we discussed the structure and function of the erythrocyte membrane skeleton in great detail. Until 1981, it was generally believed that spectrin and related molecules were restricted to the membrane of the terminally differentiated and highly specialized erythrocyte, while other cells were thought to contain a transcellular cytoskeleton including actin microfilaments, microtubules, and intermediate filaments. This misconception was based on the failure of initial attempts to demonstrate the presence of spectrin in nonerythroid cells by a complement fixation assay[264] and radioimmunoassay[265] utilizing antibodies against human RBC spectrin. Most historians of this field credit Goodman and colleagues[9,10] with the initial discovery of spectrin-related molecules in nonerythroid cells.[11,12,33,69,266-270] Despite the dogma prevalent in 1980 stating that spectrin was restricted to erythrocytes, we began our search for spectrin-related molecules in nonerythroid cells based on the detection of immunoreactive analogs of ankyrin in diverse cells and tissues[132] and a hunch that nonerythroid cells should require a macromolecular structure similar to the spectrin membrane skeleton to give structural stability and contour to their membranes. We began by demonstrating by indirect immunofluorescence that embryonic chicken cardiac myocytes (ECCM), mouse fibroblasts (3T3 cells), and rat hepatoma cells (HTC, HMOA) were specifically stained with antibodies against human RBC spectrin. While these studies demonstrated that proteins with common antigenic sites to RBC spectrin were present in these cells, it did not demonstrate that they were close relatives to the erythrocyte protein. Goodman et al.[9,10] therefore attempted to immunoprecipitate spectrin-like molecules from a neutral detergent extract of nonerythroid cells (ECCM) with anti-RBC spectrin IgG. Since our antibody against human RBC spectrin preferred the α subunit of erythrocyte spectrin, we performed the immunoprecipitation under nonstringent (no SDS) conditions so that the immunoprecipitated spectrin-related molecule would maintain its native subunit stoichiometry,

rather than reflecting the antibody specificity. Two polypeptides of 240 and 230 kDa mol wt were immunoprecipitated from ECCM, which not only resembled the spectrin subunits in size, but were also present in a 1:1 mol/mol stoichiometry. Since the immunoprecipitate also contained substantial quantities of myosin, actin, and other proteins, we separated the proteins by SDS-PAGE, and then stained them with the human RBC spectrin antibody by the gel overlay technique. The result demonstrated that only the 240- and 230-kDa subunits were stained with the RBC spectrin antibody. Therefore, we had demonstrated that two polypeptides of similar molecular weight to the erythrocyte spectrin α and β subunits were present as a 1:1 complex in ECCM and, furthermore, that these peptides shared antigenic determinants with human erythrocyte spectrin. However, because the dogma that suggested spectrin was confined to erythrocytes was very strong in 1980-1981, we knew that we needed to determine whether the ECCM 240- and 230-kDa polypeptides were structurally related to embryonic chicken RBC spectrin. Therefore, comparative one-dimensional chymotryptic peptide mapping was performed on ECCM 240- and 230-kDa polypeptides and embryonic chicken erythrocyte spectrin α and β subunits radioiodinated within the gel slice. These structural studies clearly demonstrated that the ECCM spectrin 240- and 230-kDa polypeptides were nearly identical to chicken RBC α and β spectrin subunits. These studies established that these polypeptides were not only closely related to spectrin subunits in size and stoichiometry, and also antigenically related, but were closely related structurally as well. These seminal findings were first presented at an ICN-UCLA symposium in March 1981[9] and then in complete form later that same year.[10] These studies, which opened the nonerythroid spectrin field, were rapidly confirmed and extended by many laboratories. Some of this pioneering work is discussed later. One reviewer,[269] who inexplicably claimed that our initial identification of nonerythroid spectrin was based solely on cross-reactivity of this protein with spectrin antibody, has not accurately portrayed these experimental findings.

Before discussing the studies which confirmed the discovery of nonerythroid spectrin, we discuss studies which appeared in parallel and which in retrospect added to our knowledge of the functions of nonerythroid spectrin, despite the fact that the authors did not recognize at the time that they were investigating nonerythroid spectrin. Davies and Klee[271] isolated a calmodulin-binding protein from bovine brain with subunits of 235 and 230 kDa which had the ability to bind f-actin. The protein was not immunoprecipitated with human RBC spectrin antibody, therefore it was thought not to be spectrin-related and was named calmodulin-binding protein-I (CBP-I). Kakiuchi et al.[272] purified a 240-kDa polypeptide from a 6-*M* urea extract of bovine brain, based on its ability to bind to a calmodulin affinity column. Shimo-oka and Watanabe[273] isolated two associated 240- and 235-kDa polypeptides from the microtubule associated protein (MAP) fraction of pig brain. These polypeptides had the ability to bind actin and stimulate actomyosin Mg^{2+}-ATPase. This protein was named brain actin-binding protein (BABP). Finally, Levine and Willard,[274] in an elegant characterization of two axonally transported polypeptides of 250 and 240 kDa, prepared antibodies against the guinea pig protein and demonstrated by indirect immunofluorescence that this protein doublet was highly concentrated at the internal periphery of neurons, Schwann cells, and a wide variety of nonneural cells and tissues. This axonally transported protein bound actin, but attempts to demonstrate a reaction between antibodies against this protein and guinea pig RBC spectrin by Ouchterlony analysis were negative. Since this protein was found to be electrophoretically and antigenically distinct from erythrocyte spectrin, suggesting to the authors that it was not a nonerythroid form of spectrin, Levine and Willard named it fodrin (from the Greek *fodros*, meaning lining). In retrospect, these studies taught us that brain spectrin bound calmodulin to its 240-kDa α subunit, associated with f-actin, was axonally transported, and related molecules were present in diverse nonneural cells. However, in terms of historical perspective, it is important to recognize that these authors did not know that they were studying nonerythroid spectrin.

The confirmation of our discovery of nonerythroid spectrin was rapid and compelling.

Glenney et al.[275,276] isolated a spectrin-related molecule from chicken intestinal brush borders which they named terminal web (TW) 260/240. Glenney demonstrated that TW 260/240 shared several properties with chicken RBC spectrin and chicken brain spectrin (fodrin) including: similar hydrodynamic parameters ($S_{20,w}$ = 11, R_s > 200 nm), similar morphology on rotary-shadowed electron micrographs, common antigenic sites on the 240-kDa α subunits, ability of the α subunits to bind calmodulin, and closely related one-dimensional peptide maps for the α subunits but distinct maps for the β subunits (260, 235, and 220 kDa). These results suggested that TW 260/240 and avian fodrin were members of the spectrin family of molecules and that they contained a constant α subunit and a variable β subunit. The fact that all avian spectrins had a highly related or identical α subunit explained and confirmed our finding that the ECCM 240-kDa subunit and the chicken RBC spectrin α subunit yielded nearly identical peptide maps.[9,10] Repasky et al.,[277] using an antibody against chicken α RBC spectrin, demonstrated the presence of spectrin-related molecules in various avian and mammalian cells and tissues including neurons, lens cells, endothelial and epithelial cells of the GI and respiratory tracts, skeletal and cardiac muscle, and skeletal myotubes in culture. This and subsequent studies indicated that spectrin-related molecules are ubiquitous components of nonerythroid eukaryotic cells and tissues. We previously published a comprehensive list of the many cells and tissues in which spectrin-related molecules have been found.[13] Bennett et al.[278] and Burridge et al.[279] isolated a spectrin-related molecule from mammalian brain and demonstrated that this molecule shared antigenic sites with mammalian RBC spectrin. The brain spectrin had 240- and 235-kDa subunits[279] in a 1:1 mol/mol ratio, with hydrodynamic properties similar to RBC spectrin ($S_{20,w}$ = 10.8, R_s = 240 Å, calculated M_r = 1,090,000).[278] These data suggested that brain spectrin was an $(\alpha\beta)_2$ tetramer, and rotary shadowing and electron microscopy indicated a two chain helically entwined rod-like structure with a contour length (200 nm) which was identical to the RBC spectrin tetramer.[278] Unlike the avian spectrins, one-[279] and two-dimensional[278] peptide maps of the mammalian RBC and brain spectrin indicated distinct α and β subunits. Mammalian brain spectrin was demonstrated to bind f-actin and to contain an ankyrin-binding site.[278,279] These studies demonstrated that mammalian nonerythroid spectrins were functionally, as well as morphologically and antigenically, related to mammalian RBC spectrin. To summarize, within 1 year following the discovery of nonerythroid spectrin,[9,10] we knew that spectrin-related molecules were ubiquitous, morpholically related to RBC spectrin, and able to bind actin, calmodulin, and ankyrin.

Glenney and Glenney[280] cleverly made use of the constant α subunit of avian spectrin to immunoprecipitate spectrins from various avian tissues. They then determined the identity of the variant subunits by immunoblotting with variant subunit antibodies and by two-dimensional peptide mapping of the immunoprecipitated subunit. They found that avian RBCs, skeletal muscle, and cardiac muscle contain a highly related 220- or 230-kDa β or β′ subunit. This result, as well as the studies of Nelson and Lazarides[281] demonstrating a 230-kDa β spectrin variant in chicken cardiac muscle, confirmed our initial finding that ECCM 230-kDa spectrin subunit yielded a nearly identical peptide map to chicken RBC β spectrin.[9,10] Glenney and Glenney[280] went on to demonstrate that most cell types (lymphocytes, hepatocytes, neurons, and fibroblasts) contain the 235-kDa variant subunit in avian tissues. Chicken intestinal epithelial cells contain the 260-kDa variant subunit in addition to the 235-kDa subunit, and cardiac muscle contains both the 220- and 235-kDa forms. These results indicated that in avian tissues the 240/235-kDa form of spectrin first discovered in brain is found in most cell types, while TW 260/240 is found only in the intestine, and the 240/220- or 240/230-kDa RBC-type spectrin is found also in skeletal and cardiac muscle. Although not completely understood, the situation in mammalian cells would appear to be different and perhaps more complex. For example, mammalian intestine contains the 240/235-kDa form of spectrin, but not TW 260/240.[289] Furthermore, mammalian brain contains two antigenically distinct isoforms of spectrin, both of which contain 240- and 235-kDa subunits, but which have quite different location within neuronal and glial

cells.[283] A detailed study of the location of distinct spectrin isoforms within various mammalian cells and tissues is very much needed.

Spectrin-related molecules are ubiquitous, and hundreds of papers have been published describing the location and structure of tissue-specific spectrin molecules. Rather than attempt to discuss all of the work on nonerythroid spectrin, we decided to focus instead on the structure, location and function of brain spectrin, which is the best-studied nonerythroid spectrin molecule. We conclude the discussion of nonerythroid spectrin with a short discussion on TW 260/240 to compare and contrast it to the well-characterized brain spectrin molecule. We apologize in advance to the authors of many important studies on other nonerythroid spectrins which are not included for lack of space.

B. Brain Spectrin

As described in the previous section, the spectrin-related molecules found in brain were given several descriptive names (CBP-I, BABP, fodrin, calspectin) prior to an understanding of their close relationship to erythrocyte spectrin. Most researchers in the field currently refer to the nonerythroid spectrin-related molecules by stating the tissue or cell in which they are found followed by the term spectrin (e.g., brain spectrin, liver spectrin, Hela spectrin). In tissues in which more than one spectrin analog exists, the nomenclature should include the molecular weight of the subunits ($\times 10^{-3}$) in parenthesis.[13] For example, chicken brain contains two spectrin isoforms: an erythrocyte-related brain spectrin(240/230) and a unique brain spectrin(240/235).[282] Mammalian brain presented a new problem in terms of nomenclature because two isoforms are present which both have subunits of 240 and 235 kDa. We therefore decided to refer to the isoform which was antigenically related to erythrocyte spectrin as brain spectrin(240/235E) and the unique isoform as brain spectrin(240/235).[283] In the following sections, we discuss the structure, location, and function of brain spectrin. The reader should be aware that most of the studies on brain spectrin structure and protein interactions predated the discovery of brain spectrin isoforms. Therefore many of the studies were performed with total brain spectrin preparations which were a comixture of brain spectrin isoforms. While much useful information has been derived from this type of study, all will need to be reinvestigated with the purified isoforms. The recent purification of the axonal isoform of mammalian brain spectrin(240/235) indicates that such studies are now possible.[283]

1. Structure

Brain spectrin has been isolated from guinea pig, chicken, mouse, pig, bovine, and human.[271,273-276,278,279,283-286] In most cases, the high molecular weight subunits of brain spectrin which are present in a 1:1 mol/mol ratio have been assigned molecular weights of 240 kDa (α) and 235 kDa (β).[273,275,276,279,283-286] Careful hydrodynamic studies performed on pig and mouse brain spectrin have yielded nearly identical physical parameters ($S_{20,w}$ = 10.5 to 11, R_s = 214 to 220 Å, $\bar{v}$ = 0.725 g/cc based on amino acid composition).[284,285,287] From these parameters, a molecular weight of ~972,000 Da can be calculated, along with a frictional ratio (f/fo) = 2.9. These values indicate that brain spectrin must be a highly asymmetric molecule, which takes the form of an $(\alpha\beta)_2$ tetramer.

Rotary-shadowed electron micrographs of brain spectrin demonstrate a long flexible rod of 200-nm contour length, with the two strands woven into a tight double helix with few gaps, which is associated at both ends.[14,275,278,287] The morphology of brain spectrin (illustrated in Figure 6) is nearly identical to erythrocyte spectrin tetramer (Figure 2), which is a more loosely woven double helix with a contour length of 200 nm. Brain spectrin, similar to erythrocyte spectrin tetramer, is formed by head-to-head interaction of two $\alpha\beta$ heterodimers, as indicated by the bilateral symmetry of binding sites for monoclonal antibodies against brain spectrin,[288] f-actin, ankyrin, calmodulin and synapsin I (Figure 6). The studies indicating the location of binding sites for f-actin, ankyrin, calmodulin, and synapsin I are discussed in detail in Section

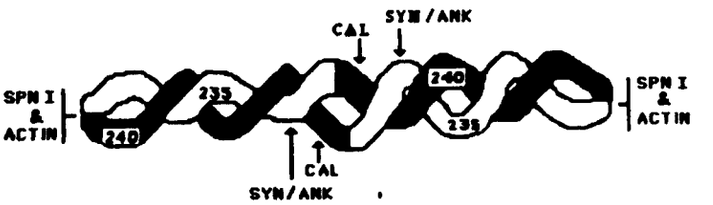


FIGURE 6. Model of the brain spectrin molecule. The morphology of the 200-nm long brain spectrin tetramer is presented, along with bivalent binding sites for calmodulin (Cal), syndein/ankyrin (Syn/Ank), actin, and synapsin I (SPN I), which is an analog of brain protein 4.1. (Taken from Goodman, S. R., Riederer, B. M., and Zagon, I. S., *Bioessays*, 5, 25, 1986. With permission.)

III.B.3. For the sake of this discussion, it is important only to recognize that when two copies of any one of these molecules are bound to brain spectrin, the binding sites are on opposite strands, equidistant from the center of the molecule. It is interesting that when α and β subunits are isolated after treatment of brain spectrin with 6 *M* urea, the subunits will reassociate into $(\alpha\beta)_2$ tetramers after removal of the urea.[287] Indeed, the association sites at the ends of the brain spectrin molecule must be related in sequence to the terminal association sites of RBC spectrin because hybrid molecules containing brain α spectrin and RBC β spectrin can be formed. These hybrid molecules retain the morphology, actin-binding capacity, and ankyrin-binding capacity of their parent molecules.[287] Yet, while the terminal association sites of brain and RBC spectrin are related,[287] they clearly are not identical. Erythrocyte spectrin, when highly concentrated at 30 to 37°C, is capable of forming oligomers larger than the $(\alpha\beta)_2$ tetramer, while brain spectrin placed under identical conditions will not oligomerize beyond the tetramer.[286]

Published structural studies on brain spectrin have thus far been limited to comparisons of their peptide maps to those obtained for RBC spectrin. Yet these studies have already indicated interesting differences between avian and mammalian spectrins. One- and two-dimensional peptide-mapping analyses of avian brain vs. RBC spectrin have indicated nearly identical α subunits, but show only limited homology between the 220- and 235-kDa β subunits.[276,289] However, one- and two-dimensional peptide-mapping analyses of mammalian spectrins indicate that both the α and β subunits of brain spectrin are quite different from α and β subunits of RBC spectrin.[278,279,284,285] It appears that α spectrin in all avian tissues is highly related and may in fact represent the product of a single gene, while the β subunits of avian RBC and brain spectrin are distinct gene products. The α subunit of mammalian brain spectrin appears more closely related to the avian α spectrins than to mammalian RBC spectrin,[289] while the 235-kDa subunit of mammalian brain spectrin yields a nearly identical peptide map to the 235-kDa subunit of avian brain spectrin.[289] Recent confirmation of these findings have come from cDNA cloning and sequencing of human lung fibroblast spectrin.[290] Several overlapping cDNA clones encompassing 2760 nucleotides of the α spectrin subunit were isolated from a human lung fibroblast cDNA library. Alignment of the composite 919 predicted amino acid sequence indicated a 106 amino acid repeat unit similar to RBC spectrin, with only 55 to 59% homology to human RBC spectrin α-7 to α-15 repeat units, but 90 to 96% homology to the same region of avian RBC spectrin. The peptide-mapping and cDNA-sequencing results collectively suggest that, following duplication of a common ancestral α subunit gene after the separation of the mammalian and avian lines, the gene encoding the mammalian RBC α subunit diverged early from the ancestral gene, while the gene encoding mammalian nonerythroid spectrins changed relatively little. However, no sequence analysis (protein or cDNA) on brain spectrin has appeared. It will be very interesting to see the comparative sequences of the mammalian brain spectrin isoforms (240/235) and (240/235E). While it has not yet been ascertained whether these

isoforms differ in terms of sequence or posttranslational modification, their relative cross-reactivity with RBC spectrin antibodies[283] would suggest that brain spectrin(240/235E) should be much closer in structure to erythrocyte spectrin than brain spectrin(240/235). It is of interest that the two spectrin cDNA clones isolated from the human lung fibroblast cDNA library[290] differed in that one contained a 60 nucleotide in-frame insertion which would result in a protein with an additional 20 amino acids between repeat units α-10 and α-11. Therefore mammalian lung fibroblasts like brain appear to contain two spectrin isoforms, which in the case of lung differ in primary sequence.

Studies carried out on total mouse brain spectrin indicated that the 235-kDa subunit is phosphorylated *in vivo* and *in vitro*.[285] Mice injected with ^{32}P-orthophosphate incorporated the label into a 235-kDa polypeptide which could be immunoprecipitated under stringent conditions with antibody against brain spectrin.[285] The same 235-kDa polypeptide was phosphorylated *in vitro* when brain membranes were incubated with [γ-^{32}P] ATP ± cAMP.[285] Therefore, analogous to RBC spectrin, brain spectrin is phosphorylated in its β subunit by a cAMP-independent protein kinase. We do not yet know the number of phosphates per brain spectrin molecule or the location of the phosphorylation sites. These studies must now be carried out on isolated brain spectrin isoforms, which could potentially differ in their state of phosphorylation. Based on the studies of Sobue et al.,[291] we must also ask whether the phosphorylation of brain spectrin isoforms is mediated by a Ca^{2+}-calmodulin protein kinase.

2. Isoforms

a. Discovery of Mammalian Brain Spectrin Isoforms

The discovery of mammalian brain spectrin isoforms began with a puzzling set of data which initially seemed contradictory. Levine and Willard prepared antibodies against an axonally transported protein with subunits of 240 and 235 kDa mol wt, which they had named fodrin.[274] Fodrin was isolated by high salt extraction of guinea pig crude brain membranes followed by gel filtration chromatography and preparative gel electrophoresis,[274] and the antibody raised against this protein cross-reacted primarily with the 240-kDa α subunit of the 240/235-kDA protein doublet.[292] Immunohistochemical analysis with this antibody detected fodrin (brain spectrin) in the cortical cytoplasm of guinea pig neuronal cell bodies, dendrites, and axons in the peripheral nervous system, as well as the plasma membrane of Schwann cells.[274] In contrast, Zagon et al.[293] used an antibody against mouse RBC spectrin, which detected 240- and 235-kDa polypeptides exclusively among total mouse brain protein, localized brain spectrin to mouse neuronal cell bodies and dendrites, but not axons. Staining of glial cell bodies with RBC spectrin antibody was also observed.[293]

These results raised the interesting conundrum of how two antibodies both specifically recognizing brain spectrin, could indicate different localizations on immunohistochemical analysis. The answer was found when Riederer et al.[283] isolated brain spectrin from an enriched fraction of mouse synaptic/axonal plasma membrane (SPM), isolated mouse RBC spectrin, and prepared antibodies against both. The antibody against synaptic/axonal spectrin, after "cleaning" on a RBC spectrin-affinity column, reacted with the 240-kDa subunit of SPM brain spectrin on immunoblots and immunodots, but did not cross-react with RBC spectrin. Conversely, antibody against mouse RBC spectrin, after cleaning through a SPM brain spectrin-affinity column, reacted with mouse RBC spectrin on immunoblots and dots, but did not cross-react with SPM spectrin.[283] Most importantly, this RBC spectrin antibody, which did not react with the 240- or 235-kDa subunits of mouse SPM spectrin, did cross-react weakly with a 240-kDa polypeptide and strongly with a 235-kDa polypeptide in the total brain homogenate. This finding indicated that there were two immunologically distinct spectrin isoforms in mouse brain: SPM brain spectrin (named brain spectrin[240/235]) which did not react with RBC spectrin antibodies and brain spectrin(240/235E) (E stands for erythrocyte-related) which could.[283] These antibodies were used to locate the two isoforms within mouse brain tissue by immunohistochemical

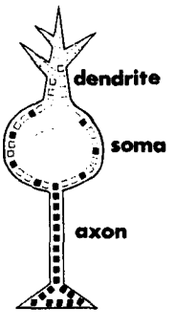


FIGURE 7. Summary of the location of spectrin isoforms within the mammalian neuronal cell. Brain spectrin(240/235E), detected with RBC spectrin antibody, is found throughout the dendrites and the cell body (❑). Brain spectrin(240/235) is located in the axon and to a lesser extent in the cell body (■). (Taken from Goodman, S. R., Riederer, B. M., and Zagon, I. S., *Bioessays*, 5, 25, 1986. With permission.)

analysis. Brain spectrin(240/235) was enriched in mouse neuronal axons and to a lesser extent in cell bodies, but was not found in dendrites or in glial cells. Brain spectrin(240/235E) was present in mouse neuronal cell bodies and dendrites, but was excluded from axons. Unlike brain spectrin(240/235), brain spectrin(240/235E) was also present in certain glial cell types.[283] A summary of the location of brain spectrin(240/235) and (240/235E) within a single neuron is presented in Figure 7.

With the discovery of two mammalian brain spectrin subtypes, the results of Levine and Willard[274] and Zagon et al.[293] could be reconciled. On isolation of fodrin (brain spectrin) by high ionic strength extraction of crude brain membranes, Levine and Willard[274] were extracting and preparing antibody against both brain spectrin isoforms. Therefore, when staining the neuronal cell body, dendrites, and axons of guinea pig peripheral nervous tissue (as well as Schwann cells) with antibody against fodrin, Levine and Willard were detecting both brain spectrin isoforms. Zagon et al., using a mouse RBC spectrin antibody, were specifically detecting brain spectrin(240/235E).[293] The results from these two laboratories were not contradictory, but simply predated our knowledge of brain spectrin isoforms and can now be properly interpreted.

We have recently demonstrated that brain spectrin(240/235) and brain spectrin(240/235E) are also present in human, pig, and rat brain,[294,295] with identical localization to that described for mouse neural tissue.[283] It is important to note that when proteolytic activity is not completely blocked during brain membrane preparation or immunoprecipitation from neutral detergent extracts of brain tissue, the 235-kDa subunit of brain spectrin(240/235) is converted to a 230-kDa polypeptide, while the 235-kDa subunit of brain spectrin(240/235E) is converted to a 232-kDa proteolytic fragment.[295] This, we believe, explains the recent suggestion by Siman et al.[296] that rat brain contains a spectrin(240/235) isoform and a (240/230E) isoform. When we repeated the immunoprecipitation or membrane preparations as performed by Siman et al.[296] without protease inhibitors, we also found an ~230E polypeptide (actually 232 kDa).[295] However, the same immunoprecipitation or membrane preparation in the presence of potent protease inhibitors, indicated that rat brain contained the same 240/235 and 240/235E isoforms found in mouse brain. Unfortunately, these authors[296] used the differential appearance of a 235- vs. a 230-kDa polypeptide in rat brain homogenates of different neural regions or times of development, to discuss the localization and ontogeny of rat brain spectrin isoforms. As is probably apparent from the preceding discussion, such an approach speaks as much to the endogenous protease activity of different brain regions or times of development as it does to the location or ontogeny of spectrin isoforms. In any case, using a fodrin antibody, Siman et al.[296] detected both spectrin

isoforms in all rat neuronal compartments, as would be expected from the results of Levine and Willard,[274] while an antibody against rat RBC β spectrin detected the erythrocyte-related isoform in rat neuronal cell bodies and dendrites, in agreement with Riederer et al.[283]

It was clear from the initial results of Riederer et al.[283] that the 235-kDa subunits of mouse brain spectrin(240/235) and (240/235E) were immunologically distinct. In addition, the fact that antibodies against the α subunit of brain spectrin(240/235) stained only axons despite the fact that brain spectrin(240/235E) was enriched in dendrites and cell bodies suggested that the 240-kDa subunits were also immunologically distinct.[283] This suggestion has been confirmed by the recent observation that antibodies which recognize only the 240-kDa subunit or only the 235-kDa subunit of brain spectrin(240/235E) both stain neuronal cell bodies and dendrites; while antibody against the 240-kDa subunit of brain spectrin(240/235) stains axons on immunohistochemical analysis of mammalian brain.[295] Therefore, both the α and β subunits of the two brain spectrin isoforms are antigenically distinct.

b. Avian vs. Mammalian Brain Spectrin Isoforms

Lazarides and Nelson[282] described two forms of chicken brain spectrin. Chicken brain spectrin(240/235) (referred to as αγ spectrin by these authors, although this nomenclature is not generally accepted) was found in neuronal cell bodies, axons, and dendrites with an antibody against chicken RBC spectrin α subunit. Unfortunately, since the α subunit of avian spectrins is constant,[276] Lazarides and Nelson would be detecting all avian spectrin isoforms. This caveat leaves the location of avian brain spectrin(240/235) unclear and suggests that the experiment requires repeating with an antibody directed against the 235-kDa subunit of the 240/235 spectrin isoform. Brain spectrin(240/230 or 240/220, αβ′ or αβ), an isoform which is antigenically and structurally nearly identical to chicken RBC spectrin, is located exclusively on the plasma membrane of the neuronal cell body and dendrites.[282] The location of the erythrocyte-related avian brain spectrin is quite similar to the neuronal compartmentation of mammalian brain spectrin(240/235E),[283] yet mammalian brain spectrin(240/235E) is structurally distinct from mammalian RBC spectrins, which all contain a 220-kDa β subunit. We would recommend great caution when extrapolating results concerning avian brain spectrin isoforms to questions concerning mammalian brain spectrin isoforms.

c. Immunoelectron Microscopic Localization of Brain Spectrin Isoforms

The mouse brain spectrin isoforms have been localized within individual neuronal and glial cells of the cerebellum by immunoelectron microscopy utilizing the immunoperoxidase technology.[297] Brain spectrin(240/235E) was concentrated in neuronal cell bodies, dendrites, and postsynaptic terminals. It was associated with the cytoplasmic surface of the plasma membrane and organelle membranes (mitochondria, endoplasmic reticulum, nuclear envelope). This isoform was also associated with neurofilaments, actin filaments, and microtubules (it ran along the axis of microtubules as well as cross-linking microtubules). Brain spectrin(240/235E) was located in the synaptic spines of dendrites and was especially associated with postsynaptic densities. Postsynaptic regions of dendrites, presumably the sites of axodendritic synapses, also contained brain spectrin(240/235E). Confirming earlier studies,[293,294] brain spectrin(240/235E) was also localized to the plasma membrane, nuclear envelope, mitochondria, endoplasmic reticulum, and cytoskeletal structures of glial cell bodies.

Brain spectrin(240/235) was detected in axons and presynaptic elements,where it was associated with the cytoplasmic surface of the plasma membrane, organelle membranes (mitochondria), synaptic vesicles, and cytoskeletal structures (microtubules, neurofilaments, and actin filaments).[257] The significance of the brain spectrin(240/235)-synaptic vesicle interaction to the early events in synaptic transmission is discussed in Section III.B.4.b. The association of brain spectrin(240/235) with mitochondria within the axon is consistent with the observation that a portion of brain spectrin moves down the axon of retinal ganglian cells at the

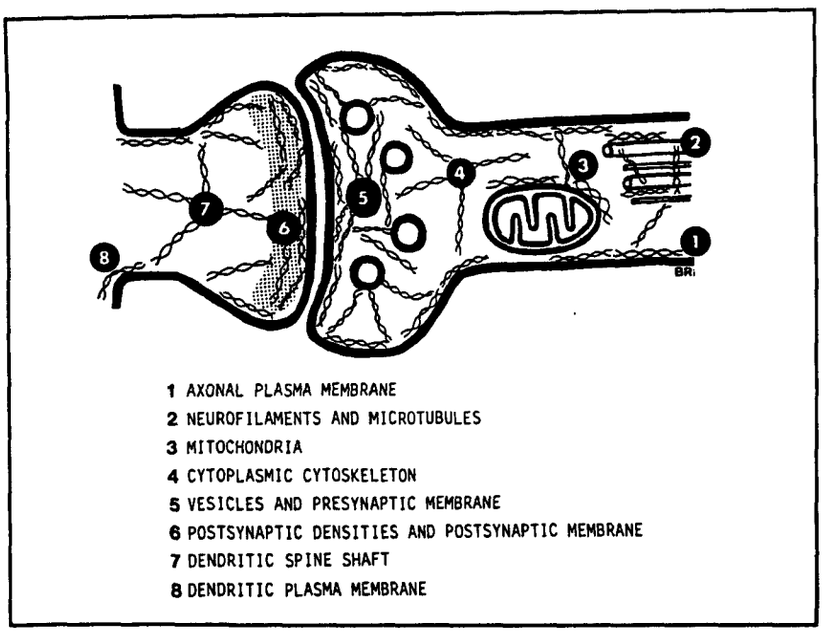


FIGURE 8. Location of spectrin isoforms at an axo-dendritic synapse. This model summarizes the location and interactions of spectrin isoforms at an axo-dendritic synapse based upon the immunoelectron microscopic study of Zagon et al.[293] *The locations of brain spectrin(240/235) are demonstrated in 1 to 5, and brain spectrin(240/235E) are shown in 6 to 8.*

same rate (40 mm/d) as mitochondria. Interestingly, brain spectrin also moves down the axon at the velocity of actin (2 to 8 mm/d) and at slow velocity (1 mm/d) along with microtubules and neurofilaments.[298] The potential role of brain spectrin(240/235) in axonal transport is discussed in Section III.B.4.a. Consistent with immunoelectron microscopy, which indicates an association of both brain spectrin isoforms with microtubules, neurofilaments, and actin filaments throughout the neuronal cytoplasm,[297] are the findings of the coisolation of brain spectrin with microtubules isolated under stabilizing conditions;[299] coisolation with tubulin and neurofilament polypeptides in a cell-free model of slow axonal transport;[300] binding to polymerized pig brain tubulin;[301] and coimmunoprecipitation with MAP1, MAP2, tubulin, actin, and neurofilament proteins from solubilized rat cerebral cortex.[302]

The location and associations of mammalian brain spectrin isoforms at the electron microscope level of resolution are summarized in Figure 8. It appears that both isoforms are either associated with membrane surfaces or are found in association with cytoskeletal structures throughout the cytoplasm of neuronal and glial cells. The discovery that two spectrin isoforms with distinctive compartmentation exist throughout the cytoplasm of neural cells, as well as in association with the plasma membrane, suggested that brain spectrin was probably a more functionally versatile protein than erythrocyte spectrin.

d. Spectrin Isoforms and Brain Development

With the discovery that at least two spectrin isoforms exist in the mammalian nervous system,[283] the relationship of these spectrin subtypes during mammalian brain development was first addressed by Riederer et al.[303,304] These workers discovered that the isoforms of brain spectrin are differentially expressed during neuromorphogenesis. Utilizing a wide range of ages, both fetal and postnatal mouse brain were examined by immunocytochemistry with antibodies

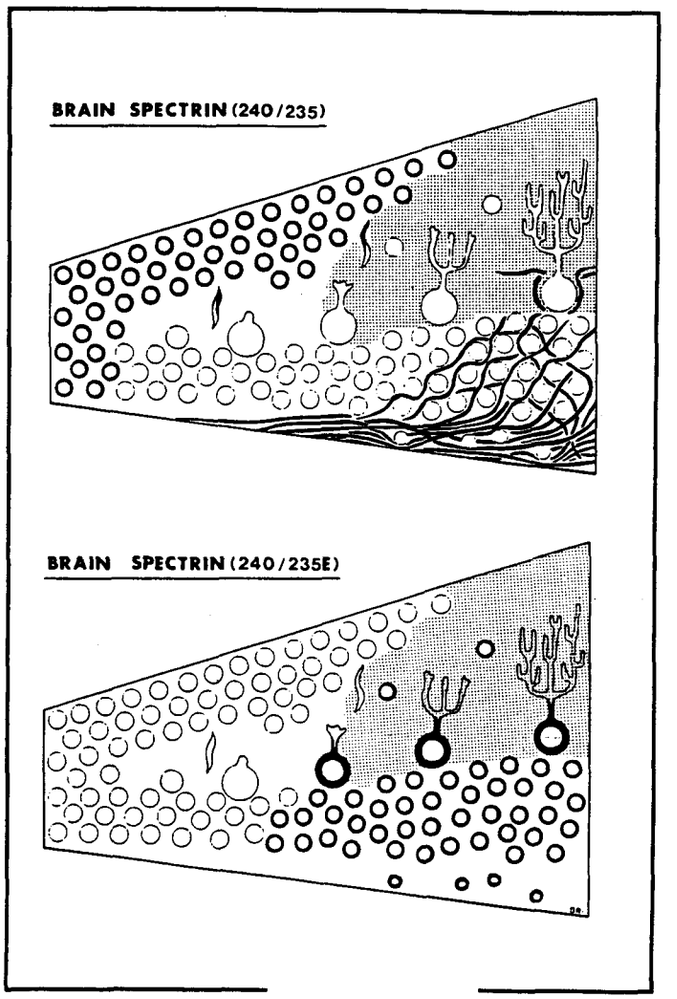


FIGURE 9. Ontogeny of brain spectrin isoforms. Schematic drawing summarizing the localization of spectrin subtypes (denoted by blackened lines) in the mammalian cerebellar cortex during ontogeny; stages of growth from fetal (far left) to adult (far right) are depicted. Brain spectrin(240/235) is found in both the neuroepithelial and external germinal cells during ontogeny. As development proceeds, this spectrin subtype is prominently seen in axons and in presynaptic areas (e.g., glomeruli in the internal granule layer). Brain spectrin(240/235E) is not detected in the fetus and initially appears in the cerebellar cortex during the second postnatal week. This subtype is located in neuronal and glial cell soma, dendrites, and postsynaptic regions. (Reprinted from Zagon, I. S., Riederer, B. M., and Goodman, R. S., *Br. Res. Bull.*, 18, 799, 1987. With copyright permission from Pergamon Journals, Ltd.)

which specifically recognized brain spectrin(240/235) and brain spectrin(240/235E). Brain spectrin(240/235) was detected in fetal brain tissues and increased twofold to adult levels. This subtype was enriched in the cortical cytoplasm of primary and secondary germinative neural cells and was also associated with fibers resembling axons in the fetus (Figure 9). Brain spectrin(240/235E), a brain subtype specifically identified with antibodies to RBC spectrin, was below the limits of detection in the fetal and neonatal brain, but rapidly increased in concentration during the second postnatal week. Brain spectrin(240/235E) was found in the cell body and

dendrites of differentiating neurons and glial cells, but was not recorded in mitotic cells (Figure 9). This subtype was especially prominent in granule neurons of the cerebellum and dentate gyrus.

The ontogeny of rat brain spectrin isoforms was documented by Siman and collaborators.[296] These investigators found that rat brain spectrin(240/235) was expressed in the neonatal brain, while the erythrocyte-related isoform first appeared after the second postnatal week. Therefore, despite the interpretive problems in the study by Siman et al.[296] previously detailed, it does appear to confirm the basic concepts described for spectrin isoform expression in developing mouse brain.[303,304] Therefore, it would appear that mammalian brain spectrin isoforms are expressed differently during neuroontogeny. Brain spectrin(240/235E) is not expressed until midway in the preweaning period of rodent brain development. By this time, neurogenesis in all areas except cerebellum, hippocampus, and olfactory bulbs is largely complete, and such processes as synaptogenesis and myelination are well underway. In terms of physical characteristics and behavioral ontogeny, both rats and mice have undergone substantial advancement by the second postnatal week.[305] Thus, the rather late onset in the expression of brain spectrin (240/235E) suggests that the functional importance of this isoform lies not in the early periods of ontogeny, but in subsequent events. Brain spectrin(240/235) is present at the origin of the nervous system, suggesting that neuroontogenic processes may rely on functional attributes of this isoform. Quite clearly, a major advancement in our knowledge about spectrins and mammalian development will be gained by elucidating the function of these isoforms during neural ontogeny.

The developmental neurobiology of spectrin has also been examined in the avian brain.[306] Chicken brain spectrin(240/235) is expressed constitutively in all neuronal cell bodies during all stages of cerebellar morphogenesis. Thus, its expression is temporally similar to mammalian brain spectrin(240/235). The erythrocyte-related chicken brain spectrin(240/230) has been detected only at the stage of synaptogenesis. Therefore, chicken brain spectrin(240/230) expression is both temporally and spatially related to mammalian brain spectrin(240/235E).

The discovery, structure, location, function, and ontogeny of brain spectrin isoforms have received previous review.[14,15,294,304]

3. Protein Interactions

In Section II of this review, the numerous protein interactions that erythrocyte spectrin participates in were described. Our current knowledge of proteins that interact with brain spectrin includes calmodulin, ankyrin, brain 4.1 (amelin and synapsin I), and actin. It is likely that additional proteins associated with other neuronal structures will be characterized as spectrin-binding proteins in the future. The localization of binding sites of the above-mentioned proteins on the brain spectrin molecule are summarized in Figure 6 and are described in detail in this section.

In the following discussion, references to brain spectrin without indication of specific isoform represent studies that were conducted with comixtures of brain spectrin isoforms, or experiments in which the isoform was not specified. In the future, it will be necessary to conduct protein-binding studies with purified spectrin isoforms in order to assess the affinity of spectrin-binding proteins to the appropriate isoform.

a. Calmodulin

The ability of brain spectrin to bind calmodulin was its earliest demonstrated protein interaction,[271,272] preceding knowledge that the 240- and 235-kDa polypeptides were brain analogs of erythrocyte spectrin. Subsequent studies have demonstrated that brain spectrin is a major calmodulin-binding protein in brain, and that calmodulin binds to the 240-kDa α subunit of brain spectrin in a Ca^{2+}-dependent manner.[276,307] Rotary shadowing and electron microscopy of the brain spectrin-calmodulin complex indicate that the calmodulin-binding sites on both α

spectrin subunits are a distance of 15 nm from the junction of the heterodimers.[308] Avian erythrocyte spectrin shares the property of calmodulin-binding sites on the α subunit with both avian and mammalian brain spectrin.[11,276] However, as discussed in Section II.B.1.c, the mammalian erythrocyte spectrin α subunit does not bind calmodulin when tested by the gel overlay method, but a weak interaction between calmodulin and the β IV domain of human erythrocyte spectrin has been demonstrated.[120] As previously discussed, this interaction may modulate the mammalian erythrocyte spectrin-4.1-actin ternary complex.[120] It is clear that the binding of calmodulin to brain spectrin, which is approximately 85 nm removed from the actin-binding domain,[308] must play some other as yet unspecified function.

b. Ankyrin

Prior to the discovery of brain spectrin, Bennett demonstrated the presence of an immunoreactive ankyrin analog in brain by radioimmunoassay.[132] The protein exists as three sequence-related polypeptides with apparent molecular weights of 220, 210, and 150 kDa,[309] which cross-react with antibody against erythrocyte ankyrin. Isolated pig brain ankyrin binds to brain spectrin with a K_D of 25 n*M* and a stoichiometry of two ankyrin molecules per spectrin tetramer.[310] Brain ankyrin binds to the 235-kDa β subunit at sites that are ~20 nm from the junction of the brain spectrin heterodimers (Figure 6).[310] The chymotryptic digestion of brain ankyrin results in a 72-kDa spectrin-binding domain and a 93-kDa membrane-bound domain[309] similar to the domain structure described in Section II.B.2.a for erythrocyte ankyrin.[136,137] The affinity, stoichiometry, and subunit specificity of the brain ankyrin-spectrin interaction are remarkably similar to their RBC counterparts. The fact that brain ankyrin can bind to the 43-kDa cytoplasmic domain of RBC band 3 in solution[310] tempts one to speculate that the binding of brain spectrin to neural cell membranes may resemble the RBC spectrin-ankyrin-band 3 interaction. Yet caution is necessary because, despite the presence of 69- and 60-kDa immunological analogs of band 3 in neuroblastoma cells,[311] no demonstration of a functional band 3 analog in neural tissue has appeared. Furthermore, as is described later, recent evidence suggests that brain spectrin may be associated with the neuronal plasma membrane through a direct or indirect (via ankyrin) interaction with the 180-kDa neural cell adhesion molecule ($N\text{-}CAM_{180}$).

c. Actin

As discussed in Section II.B.1.b, erythrocyte spectrin tetramers are capable of cross-linking f-actin filaments (protofilaments of ~13 actin monomers) into a two-dimensional meshwork.[21] The erythrocyte spectrin/f-actin interaction is relatively weak in the absence of erythrocyte protein 4.1, but in the presence of protein 4.1 the ternary complex formation is stimulated, and a strong spectrin/4.1/f-actin complex is formed ($K_D = 10^{-12}$ *M*).[71]

Several laboratories have demonstrated the ability of brain spectrin to bind to f-actin by sedimentation and low shear viscometry methods.[273,274,278,279,312] Low-angle rotary shadowing of the brain spectrin/f-actin complex indicates that brain spectrin binds end-on to f-actin, cross-linking actin filaments analogous to the erythrocyte spectrin tetramer.[274,312]

These studies have led to the reasonable, but as yet unproven, expectation that the brain spectrin isoforms are involved in linking f-actin to the plasma membrane and organelle membranes.

d. Amelin

Following the discovery of brain spectrin, it was shown that erythrocyte 4.1 was capable of stimulating the relatively weak brain spectrin/f-actin interaction,[313,314] and this suggested that there might be a brain analog to erythrocyte protein 4.1. Using antibodies against erythrocyte 4.1, an immunoreactive and structural analog (based upon peptide-mapping analysis) was identified in mammalian brain.[315] The brain 4.1 analog, termed amelin (from the Greek *amelew*, meaning to overlook), was originally described as an 87-kDa polypeptide in continuous SDS-

PAGE systems, but migrates as a single polypeptide of 93 kDa in discontinuous SDS-polyacrylamide gels.[316-318] Indirect immunohistochemical studies with antibody against erythrocyte 4.1 have localized amelin in the cell bodies and dendrites of neurons as well as certain glial cells,[315,316] a localization that corresponds to brain spectrin(240/235E) and may reflect a difference in affinity of brain spectrin isoforms for amelin. Two-dimensional tryptic or chymotryptic iodopeptide-mapping analysis has revealed 50% spot homology between erythrocyte 4.1 and amelin.[315,316] Amelin which has been subjected to two-dimensional gel electrophoresis and transferred to nitrocellulose is capable of binding ^{125}I-brain spectrin.[316] The brain spectrin used in these assays was extracted from total brain membrane, and therefore represents a comixture of brain spectrin(240/235) and brain spectrin(240/235E).[283] It remains to be demonstrated whether the brain spectrin isoforms have different affinities for amelin and whether amelin can stimulate the brain spectrin-f-actin association in neuronal cell bodies and dendrites as well as glial cell bodies.

e. Synapsin I

It has been suggested that the previously described phosphoprotein synapsin I is an analog to erythrocyte protein 4.1 and is capable of binding to brain spectrin immobilized on nitrocellulose paper.[319] Subsequent studies have shown that, while synapsin I is structurally dissimilar to erythrocyte 4.1, it is a functional analog to erythrocyte 4.1 with respect to its spectrin-binding properties.[322]

Synapsin I is a neuron-specific phosphoprotein that is associated with the cytoplasmic surface of small (40 to 60 nm) synaptic vesicles.[320] Early studies by Greengard and co-workers have demonstrated that synapsin I is associated almost exclusively with the presynaptic region of the neuron.[321] Given the nearly exclusive localization of synapsin I in the synaptic terminal of the neuron, it is hard to reconcile the cell body and dendrite distribution of amelin if amelin and synapsin I are identical.[315,319] It is our hypothesis that, analogous to the earlier described brain spectrin isoforms, brain protein 4.1 exists as a family of subtypes, of which amelin is localized in neuronal cell bodies and dendrites, while synapsin I is localized in synaptic terminals.

Synapsin I is composed of two polypeptides of 76 and 70 kDa (Ia and Ib) mol wt, which give nearly identical two-dimensional peptide maps independent of which mammalian species is the source of material.[14] In two-dimensional chymotryptic iodopeptide-mapping analysis, synapsin I shares less than 14% peptide spot homology with erythrocyte 4.1.[14,322] Binding assays, in which synapsin I has been immobilized on nitrocellulose paper, have shown that ^{125}I-brain spectrin binds to synapsin I. Preliminary studies have suggested that brain spectrin binds preferentially to synapsin Ia relative to synapsin Ib.[316] This result indicates that the spectrin-binding site on the synapsin I molecule may be localized to the C-terminal end of the synapsin Ia polypeptide, based upon recent sequence data for synapsin Ib.[323] Dephospho-synapsin I in solution has been shown to bind to brain spectrin(240/235) with a K_D of 700 n*M* and a stoichiometry of 4 mol synapsin I per mole spectrin tetramer.[322] The binding of erythrocyte 4.1 to erythrocyte spectrin dimer has a K_D of ~200 n*M* and a stoichiometry of 2 mol 4.1 per mole spectrin dimer,[50] an affinity and stoichiometry similar to the synapsin/brain spectrin(240/235) interaction.

As described earlier, synapsin I is a phosphoprotein, and studies with a number of protein kinases have shown that the protein is differentially phosphorylated by calcium/calmodulin-dependent protein kinase II and cAMP-dependent protein kinase.[324] cAMP-dependent protein kinase phosphorylates predominantly site 1 of a 10-kDa V8 protease fragment, whereas calcium/calmodulin-dependent protein kinase II phosphorylates sites 2 and 3 in a 30-kDa V8 protease fragment.[324] The significance of synapsin I phosphorylation is that, in the resting state neuron, synapsin I is in the dephosphorylated state and upon depolarization of the neuron synapsin I becomes phosphorylated.[325] Recent studies have indicated that synapsin I, which is phosphorylated at sites 2 and 3 in a collagenase-sensitive tail region, may bind with lower affinity (K_D = 50 n*M*) to small synaptic vesicles, whereas dephosphorylated synapsin I, or

synapsin I which is phosphorylated at site 1 in a collagenase resistant globular head region, binds to small synaptic vesicles with higher affinity (K_D = 10 n*M*).[326] It remains to be demonstrated whether this slight difference in affinity of synapsin I for the synaptic vesicles represents a physiologically relevant modulation, given the estimated mean concentration of synapsin I in brain (>10^{-5} *M*).[326]

Our laboratory has demonstrated that synapsin I is capable of stimulating the brain spectrin(240/235)/f-actin interaction, as measured by a low shear falling ball viscometry assay,[327] and this interaction appears to be regulated by the phosphorylation state of synapsin I.[328] Dephosphorylated-synapsin I stimulated an eightfold increase in the apparent viscosity of brain spectrin(240/235) and preformed f-actin over that of brain spectrin and f-actin or synapsin I and f-actin alone.[328] Synapsin I which has been phosphorylated by Ca^{2+}-calmodulin-dependent protein kinase II (1.7 mol phosphate per mole synapsin I) produces no increase in the apparent viscosity of spectrin/f-actin solutions.[328] Recently, it has been shown by low-angle rotary shadowing and electron microscopy that brain spectrin(240/235) binds end-on to small synaptic vesicles (Figure 10),[318] with a K_D of ~280 n*M* through an interaction with synapsin I.[385] The significance of these studies to our understanding of the molecular events regulating synaptic transmission is discussed in Section III.B.4.b.

4. Functions

The static views of brain spectrin isoforms within individual neuronal and glial cells supplied by immunoelectron microscopy, as well as the studies of its protein and membrane interactions *in vitro* described earlier, have led to our initial understanding of brain spectrin function. Based on our detailed knowledge of the erythrocyte spectrin membrane skeleton, we can predict that the brain spectrin isoforms which are bound to the cytoplasmic surface of the plasma membrane and organelle membranes will function in (1) giving stability to these membranes, (2) regulating their contour, (3) controlling the flip-flop of phospholipids across the bilayer, and (4) limiting the lateral mobility of integral membrane proteins through the bilayer. While the first three suggestions are reasonable extrapolations from the erythrocyte model, we now have our first experimental evidence that the fourth suggestion may be correct. We describe in the following sections studies which indicate that brain spectrin (probably the 240/235E isoform) limits the lateral mobility of the neural cell adhesion molecule ($N\text{-}CAM_{180}$).

The brain spectrin isoforms found in the cytoplasm of neurons and glial cells in association with cytoskeletal structures, as well as organelles and vesicles, have additional functions. It appears that cytoplasmic brain spectrin is involved in regulating the translocation of organelles and vesicles through the three-dimensional cytoplasmic matrix. We discuss later evidence which suggests the involvement of brain spectrin(240/235) in axonal transport and the early events of synaptic transmission.

a. Axonal Transport

Brain spectrin is synthesized in the neuronal cell body, and therefore brain spectrin which has been localized in axons and presynaptic terminals[283] must be translocated from the cell body to the synapse by the process of axonal transport.

Studies on the rate of protein transport in axons have been carried out by labeling proteins biosynthetically with radioactive amino acids (such as ^{35}S-methionine) and following the appearance of labeled proteins in the axons by electrophoresis and autoradiography. Experiments of this type by Willard and co-workers demonstrated that five different populations of proteins, referred to as group I to V, travel through the axon at different velocities.[298,329,330] Group I polypeptides are the most rapidly transported (>240 mm/d) and primarily represent membrane-associated proteins.[298,331,332] Group II proteins traverse the axon at ~40 mm/d and include proteins associated with the mitochondria, which appear to represent the axonal transport of mitochondria.[298] Groups III and IV are transported at 2 to 8 mm/d and are comprised of

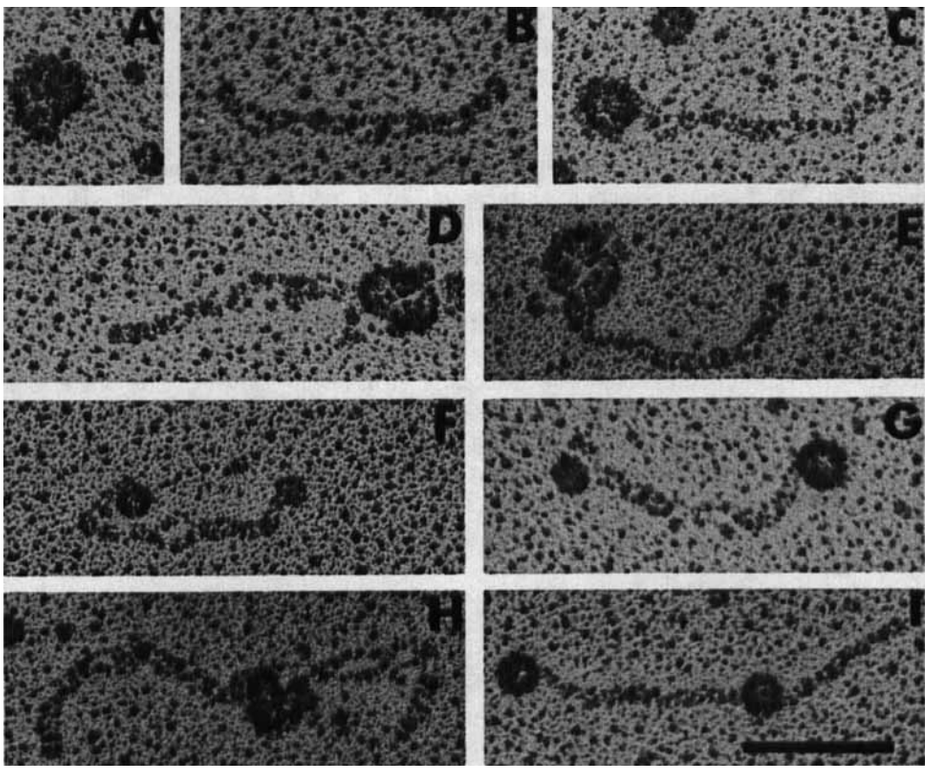


FIGURE 10. Interaction of brain spectrin(240/235) with synaptic vesicles. Purified small synaptic vesicles bind to the terminal ends of the brain spectrin(240/235) tetramer, as determined by low-angle rotary shadowing and electron microscopy. Small synaptic vesicle purified from bovine brain (A); bovine brain spectrin(240/235) tetramer (B); small synaptic vesicle bound to the terminal end of brain spectrin(240/235) (C to E); brain spectrin(240/235) cross-linking two small synaptic vesicles (F,G); two brain spectrin(240/235) tetramers binding end-on to a single small synaptic vesicle (H); two brain spectrin(240/235) tetramers cross-link two small synaptic vesicles in an extended complex (I). *Bar = 100 nm.*

cytoplasmic matrix proteins[333,334] and proteins associated with the cytoskeleton,[292,334] including actin, myosin, clathrin, calmodulin, and glycolytic enzymes.[334-339] Group V, with a velocity of 1 mm/d, is comprised of neurofilament proteins and tubulin.[330,340] This group may include movement of the cytoskeleton and associated neurofilament proteins.

There is evidence that brain spectrin (presumably brain spectrin[240/235]) travels down the axon at various rates, suggesting that there are distinct populations of brain spectrin associated with neuronal structures.[298,341] ^{35}S-methionine-labeling studies have demonstrated that brain spectrin is transported at the velocities of groups II, III to IV and V,[298,342] and sucrose gradient centrifugation studies have further shown brain spectrin traveling at the rate of group V is associated with high density structures.[298] These observations are in agreement with electron microscopic studies which have demonstrated that brain spectrin(240/235) is associated with mitochondria (group II), cytoplasmic matrix proteins (group III to IV), neurofilaments and microtubules (group V).[297]

An explanation for the movement of brain spectrin at different rates is that proteins moving at similar velocities are being transported as a unit. An example of such a relationship among transported proteins is the movement of mitochondria down axons as a unit.[298] The transport of brain spectrin has been compared to the transport of other neural proteins with which it is known to interact in an attempt to determine whether the different velocities during axonal transport are the result of transport as a complex

Actin, a brain spectrin-binding protein, has been demonstrated to be transported at a rate of 3 to 5 mm/d, the velocity of group IV proteins.[334] It has been suggested that brain spectrin, translocated at the rate of group III to IV proteins, is associated with actin and myosin in a force-generating unit that is concentrated near the cytoplasmic face of the axolemma and that this complex is translocated down the axon as a unit.[341] It is thought that f-actin-myosin acts as the force-generating unit of the complex, with brain spectrin(240/235) serving as a link between other components of the axon and the contractile unit. This process has been likened to that of lymphocyte capping, and it has been noted that the protein composition of group IV and V are similar to the proteins that co-cap with surface proteins of lymphocytes.[341]

The demonstration that brain spectrin is associated with mitochondria[297] and small synaptic vesicles[297,318] suggests that spectrin associated with these structures represents the movement of spectrin with group II polypeptides. It has been reported that synapsin I, a protein associated with the cytoplasmic surface of synaptic vesicles, is transported at various rates, the most rapid population being translocated at 30 mm/d, consistent with group II movement.[341]

Recently, a particulate fraction of crude calf brain microtubule preparations has been isolated, which when combined with purified polymerized tubulin causes ATP-dependent gelation-contraction *in vitro*.[340] The particulates had microtubule-stimulated ATPase activity and moved slowly (~1 μm/min) along microtubule walls in the presence of ATP. This particulate fraction essential for gelation-contraction, which is thought to be the cell-free equivalent of slow (group V) axonal transport, contains brain spectrin as a major component in addition to tubulin and neurofilament proteins. This represents suggestive evidence for an important role of brain spectrin(240/235) in slow axonal transport.

b. Synaptic Transmission

The role of the neuronal cytoskeleton in the regulation of synaptic transmission has only recently begun to be appreciated. Among the most significant recent advances in the study of the neuronal cytoskeleton has been the demonstration that synapsin I, a previously described synaptic vesicle phosphoprotein of unknown function, is a functional analog of erythrocyte protein 4.1 with respect to its spectrin-binding properties.[317-319,322,327] Subsequent studies have suggested that the release of synaptic vesicles from the synaptic cytoskeletal meshwork is triggered by the phosphorylation of synapsin I by calcium calmodulin-dependent protein kinase II.[317,318,322,327,328,344] Recent evidence from our laboratory has suggested that the association of brain spectrin with synaptic vesicles through an interaction with synapsin I is a dynamic relationship, regulated by changes in the phosphorylation state of synapsin I.[318,328] Thus, the convergence of the membrane skeleton and neurobiology fields has resulted in a better understanding of the role of the cytoskeleton in neuronal function. Recent discoveries in these convergent fields suggest a potential mechanism for synaptic transmission and the part that the cytoskeleton might play in this dynamic process.

Historically, theories on the mechanism by which acetylcholine is released in response to nerve stimulation was first based upon morphological studies of the synapse. The earliest descriptions of the synaptic ultrastructure at the electron microscope level demonstrated that the pre- and postsynaptic membranes are separated by a synaptic cleft 10 to 20 nm across and that a large number of vesicles ranging in size from 10 to 140 nm in diameter are present in the presynaptic cytoplasm in close apposition to the presynaptic membrane.[345-349] The term "synaptic vesicle" was first used in the literature by De Robertis and Bennett to describe the vesicles observed in an electron microscopic study of the frog sympathetic ganglia.[349] This observation, in conjunction with the earlier demonstration by Katz and colleagues that acetylcholine was released in quantal packages at the neuromuscular junction,[350,351] led to the postulation of the "vesicle hypothesis".[352]

The major tenet of this hypothesis was that each synaptic vesicle represented a discrete quantum of acetylcholine (where a quantum was thought to contain 10^3 to 10^4 molecules of

acetylcholine) and may be released in large numbers from the presynaptic terminal in response to nerve stimulation, with the resultant generation of an endplate potential.[352,353] The major steps in the release of neurotransmitter in this model would be (1) release of the vesicle, (2) vesicle translocation, (3) prefusion, and (4) fusion with the presynaptic membrane and release of neurotransmitter.

A wide range of vesicle types have subsequently been identified in synaptosomal fractions, including spherical agranular vesicles, flattened agranular vesicles, dense core vesicles, and coated vesicles. Agranular vesicles containing classical neurotransmitters vary in both size and number within the synaptosomes, with reported diameters ranging between 10 to 140 nm, and 70% of the vesicles being between 30 to 80 nm in diameter.[354] Electron microscopic studies of purified synaptic vesicles have demonstrated the presence of connections between vesicles and vesicles with "tails".[354,355] Whittaker postulated in 1966 that the fibrillar material observed in connection with these vesicles was a collapsed system, which opens during neural stimulation to release the neurotransmitter.[355] Recent revelations concerning the organization of the neural cytoskeleton add support to this theory, as discussed in the following hypothesis.

Important advances in the understanding of the role of synaptic vesicles in synaptic transmission were the identification and characterization of proteins associated with the membrane of the synaptic vesicle and the characterization of the neural spectrin cytoskeleton in the presynaptic terminal of the neuron. Wagner and Kelly characterized the physical and chemical properties and described the organization of proteins of cholinergic synaptic vesicles.[356,357] Greengard and colleagues described the neuron-specific phosphoprotein synapsin I, which is associated with the cytoplasmic surface of small (40 to 60 nm in diameter) synaptic vesicles,[320,321] and demonstrated that the phosphorylation of synapsin I in response to neural stimulation is correlated with translocation of synaptic vesicles to the presynaptic membrane and the release of neurotransmitter.[344,358] Synapsin I is phosphorylated *in vitro* and *in vivo* by a Ca^{2+}-calmodulin dependent protein kinase and by a cAMP-dependent protein kinase, and the role of synapsin I in synaptic transmission is thought to be mediated through changes in the intracellular Ca^{2+} and cAMP concentration.[325] The demonstration that synapsin I is capable of binding brain spectrin[317,322] and brain spectrin binds to synaptic vesicles via synapsin I[318,343] suggests a role for brain spectrin(240/235) in regulating the release of synaptic vesicles following stimulation of the neuron.

It is our hypothesis that in the resting state neuron, where synapsin I is thought to be in the dephosphorylated state, the small synaptic vesicles would be cross-linked via synapsin I in a three-dimensional meshwork of brain spectrin(240/235) and f-actin.[318,327,328,359] Under these conditions, the synaptic cytoplasm would be a viscous, pseudoplastic gel, preventing any appreciable diffusion of the synaptic vesicles. Upon stimulation of the neuron, the increase in the intracellular concentration of Ca^{2+} would activate the Ca^{2+}-calmodulin-dependent protein kinase present in the synapse, and the resultant phosphorylation of synapsin I at sites 2 and 3 would cause: (1) the dissociation of synapsin I (and the synaptic vesicles) from the brain spectrin/f-actin network,[328] and (2) a transient decrease in the viscosity of the synaptic cytoplasm that would facilitate the diffusion and/or active movement of the released vesicles toward the presynaptic membrane.[317,318,328] The key points of the hypothesis are outlined in Figure 11.

Preliminary calculations of the diffusion rate for a synaptic vesicle 60 nm in diameter using the Stokes-Einstein equation, which relates the translational diffusion coefficient to particle diameter and cytosolic viscosity, reveal that for a cytosolic viscosity of 5 cp[360] a vesicle located 10 nm from the presynaptic membrane would be capable of diffusing to the membrane in 0.4 ms. It has previously been demonstrated that the earliest fusions with the presynaptic membrane occur within 2.5 ms,[361] a value that is in agreement with a translational diffusion process.

Adding support to this hypothesis are studies in which dephosphorylated synapsin I was microinjected into the presynaptic terminal of the squid giant synapse which resulted in a decreased amplitude and rate of rise of the postsynaptic potential upon nerve stimulation,

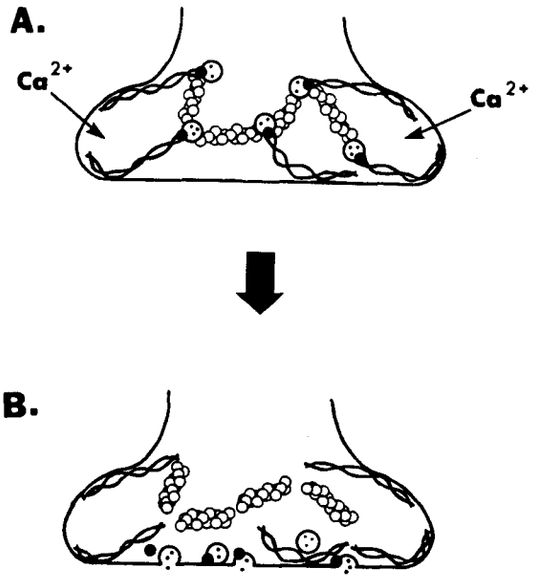


FIGURE 11. The cytoskeletal-mediated release hypothesis. Hypothetical model for the role of brain spectrin(240/235) in synaptic transmission. In the resting state neuron (A), brain spectrin(240/235) and F-actin anchor small synaptic vesicles, through dephosphorylated synapsin I, to the neuronal cytoskeleton. Upon stimulation of the neuron, calcium enters the presynaptic terminal and stimulates Ca^{2+}-calmodulin-dependent protein kinase II, which phosphorylates synapsin I. Spectrin(240/235) and F-actin become dissociated from phosphorylated synapsin I, resulting in the release of synapsin I-containing synaptic vesicles from the spectrin/actin network, and a decrease in the viscosity of the synaptic cytosol, rendering the small synaptic vesicles free to diffuse. The small synaptic vesicles translocate (either by a translational diffusion process or aided by an active mechanism) toward the active zone of the presynaptic plasma membrane and fuse with the presynaptic membrane releasing neurotransmitter (B).

whereas microinjection of Ca^{2+}-calmodulin-dependent protein kinase II produced an increased rate of rise and amplitude with a decreased latency of the postsynaptic potential.[325] While Greengard and colleagues suggest that the release of synaptic vesicles might be dependent upon the dissociation of synapsin I from the vesicle surface,[326] it is not clear why dissociation would occur with a $K_D = 10^{-8}$ *M* for the synapsin I-synaptic vesicle interaction and a synaptic concentration of synapsin I $>10^{-5}$ *M*. We believe that a more likely regulatory mechanism would be the phosphorylation-dependent dissociation of brain spectrin(240/235) and the synaptic vesicles, where the K_D for the spectrin-dephosphorylated synapsin I is ~700 n*M*.[322] This model (Figure 11), which we named the cytoskeletal-mediated release hypothesis, describes the events leading up to prefusion of synaptic vesicles with the presynaptic plasma membrane.

It has been demonstrated that synapsin I is capable of binding to membrane-binding sites independent of synaptic vesicles. The membrane-binding sites are saturable, are of a high affinity with a $K_D = 10$ n*M*, and are sensitive to protease treatment.[362] It has been suggested that synapsin I may promote the cross-linking of small synaptic vesicles to brain membranes and that this synapsin-mediated cross-linking is decreased upon phosphorylation of synapsin I by Ca^{2+}-calmodulin-dependent protein kinase II.[363] Therefore, it is possible that synapsin I also plays a

role in the pre-fusion and/or fusion events of synaptic transmission, possibly directing the binding of small synaptic vesicles to a specific docking protein in the active zone of the presynaptic membrane.

c. Regulation of the Lateral Mobility of N-CAM$_{180}$

The discussion of the preceding two sections suggests that brain spectrin(240/235) may be involved in regulating the movement of organelles, vesicles, and cytoskeletal structures within the three-dimensional cytoplasmic space of the axon and presynaptic terminal. In this section, we discuss a recent study which suggests that membrane-associated brain spectrin is also capable of regulating the lateral mobility of an integral membrane protein within the two-dimensional space of the neuronal plasma membrane.

A major property of the erythrocyte spectrin membrane skeleton is that it controls the lateral mobility of integral membrane proteins in the bilayer, thereby contributing to the surface topography of the plasma membrane. The neural cell adhesion molecule (N-CAM) has been implicated in morphogenesis of neural and nonneural tissues.[364,365] N-CAM consists of three integral membrane glycoproteins of 180, 140, and 120 kDa in adult mouse brain. The extracellular N-terminal domains of these glycoproteins share common sequence, while the three proteins differ in the length of their cytoplasmic C-terminal end. N-CAM$_{180}$, the molecular form with the largest cytoplasmic domain, is accumulated at sites of cell-cell contact and has a lower lateral mobility within the neuronal plasma membrane than N-CAM$_{140}$.[366] This has led to the suggestion that the lateral mobility of N-CAM$_{180}$ might be restricted by an interaction of its C-terminus with the cytoskeleton.[366] Pollerberg et a1.[367] recently demonstrated that brain spectrin and ankyrin coisolate with N-CAM$_{180}$ from adult mouse brain, but not with N-CAM$_{140}$ or N-CAM$_{120}$. Interestingly, brain spectrin binds directly to N-CAM$_{180}$ (but not N-CAM$_{140}$ or N-CAM$_{120}$) in a solid phase binding assay.[367] This suggests that brain spectrin can bind directly to N-CAM$_{180}$, although future studies will need to test for a possible role of brain ankyrin. Recent studies have suggested that N-CAM$_{180}$ interacts directly with brain spectrin(240/235E).[386] Taken together, these results suggest that an interaction between the cytoplasmic domain of N-CAM$_{180}$ and brain spectrin(240/235E) limits the lateral mobility of this essential adhesion molecule, restricting it to regions of cell-cell contact. This is the first demonstration of a function of brain spectrin which is analogous to the role of spectrin on the erythrocyte membrane.

d. The Biochemistry of Memory

The brain locus of memory storage has been postulated to be the neuronal synapse, and recent evidence has demonstrated that brief periods of activity in hippocampal and cortical pathways result in increased postsynaptic potential lasting for months.[368] The maintenance of a lasting postsynaptic potential is referred to as long-term potentiation.

Lynch and colleagues[369-373] postulated that the degradation of brain spectrin ! y calpain I, a calcium-activated proteinase, and an increase in the number of accessible postsynaptic glutamate receptors following synaptic stimulation are associated with long-term changes in postsynaptic structure and memory storage. Their studies have demonstrated that high-frequency afferent stimulation of hippocampal slices results in an increased number of glutamate receptors, rather than an increase in glutamate affinity,[374] and this induction of receptors was correlated with long-term potentiation. The demonstration that the potentiation effect is reduced in both size and frequency of occurrence in hippocampal slices that are maintained in a low-calcium medium[375] has suggested that a calcium-activated neutral proteinase (calpain) is involved in the regulation of glutamate receptor binding. Direct evidence of a role for calcium-activated proteinases in the regulation of the glutamate receptor was provided by the identification and partial purification of an enzyme present in rat brain synaptic membranes which, by several criteria, was demonstrated to be identical to calpain purified from other tissues.[376]

Subsequent to the discovery of calpain associated with the synaptic membrane, membrane substrates for this class of enzyme were sought. By incubating membranes in the presence or absence of calcium, and separation of membrane proteins on polyacrylamide gels, it was demonstrated that a protein doublet of 240 and 230 kDa, identified as brain spectrin, was reduced in the membrane preparations treated with calcium.[377] The conditions of membrane treatment that increased glutamate receptor binding were found to correlate well with increased degradation of brain spectrin.[377] Based upon these observations, Lynch et al. hypothesized that brief periods of high-frequency neuronal stimulation produce a transient elevation of calcium in dendritic spines which activates a membrane-associated calpain.[370] It has been suggested that the activation of calpain produces a localized breakdown of a portion of the brain-spectrin network, exposing glutamate receptors which were previously masked.[370]

While this hypothesis appears quite attractive, caution should be exercised in the interpretation of these observations. An important assumption in the hypothesis is that potentiation results from an alteration of the postsynaptic spectrin network. Unfortunately, these studies were performed using P_2 fractions which consist of crude synaptic plasma membranes containing both pre- and postsynaptic elements. Therefore, one cannot be certain that the degradation of the spectrin network is a pre- or postsynaptic phenomenon. A stronger argument for this hypothesis could be made if the studies had been carried out with purified postsynaptic densities. Interestingly, a recent immunohistochemical study with antibodies against calpain I and calpain II has demonstrated that calpain I is located primarily in neuronal cell bodies and proximal dendrites, whereas calpain II is located in axonal and glial elements,[378] a distribution that resembles the brain spectrin isoform distribution.[283] In the future, it will be necessary to examine the ability of the spectrin isoforms to act as substrates for the different calpain isozymes and to determine which spectrin isoform, if any, is associated with long-term potentiation and the unmasking of glutamate receptors.

C. TW 260/240

Microvilli contain a core of actin filaments which serve as a mechanical stiffener. These cellular extensions cover the surface of many kinds of epithelial cells, especially in tissues where cellular function requires a maximum surface area for absorption, such as in the intestine and kidney. There are about 20 actin filaments within each microvilli. The actin filaments all have uniform polarity, with their barbed ends embedded in a dense plaque of unknown composition at the microvillus tip. The core of actin filaments are attached to the plasma membrane through lateral cross-bridges which are composed of a complex between a 110-kDa protein and calmodulin. The actin filaments are bundled in the core through the cross-linking properties of villin and fimbrin. The portion of the core filaments which descends into the cytoplasmic terminal web region is referred to as core rootlets. Within the terminal web is an interrootlet zone having interdigitating thin filaments of 5 and 8 nm which cross-link adjacent core rootlets. As is discussed in detail later, the 5-nm filaments are thought to represent an intestinal form of spectrin, while the 8-nm filaments are primarily myosin. At the level of the zonula adherens, there is a circumferential bundle of actin filaments, and below the zonula adherens is the macula adherens, which has associated with it a dense meshwork of 10-nm cytokeratin intermediate filaments coursing through the basal level terminal web region, often contacting core rootlets. The terminal web region is thought to create the tension required to maintain the microvilli in a parallel upright orientation. This material has recently been discussed in a comprehensive review.[379] In the current review, we focus only on the spectrin-related molecules found in the intestinal epithelial cells.

1. Structure

Avian intestinal epithelial cells contain a unique spectrin-related molecule referred to as TW 260/240 in addition to the more general 240/235 form of nonerythroid spectrin.[11,275,280] Glenney

et al.[275] first isolated TW 260/240 by a high ionic strength extraction of chicken brush borders, followed by ammonium sulfate precipitation and gel filtration chromotography. The purified protein contained a 260-kDa β subunit and a 240-kDa α subunit in a 1:1 mol/mol ratio. The hydrodynamic properties ($S_{20,w}$ = 11, R_s > 200 nm) of TW 260/240 are similar to RBC and brain spectrin and are indicative of a highly asymmetric molecule.[275]

Rotary shadowing and electron microscopy indicated that TW 260/240 was double stranded, with the strands loosely coiled along their length and tightly associated at their ends.[275,380] The molecule, which clearly had a spectrin-related morphology, had a longer contour length (240 to 260 nm) than either RBC or brain spectrin (~200 nm).[275,380] The morphology, subunit ratio, and hydrodynamic properties of TW 260/240 all suggest that, like brain and RBC spectrin, TW 260/240 is an $(\alpha\beta)_2$ tetramer. However, at salt concentrations at or above 150 m*M*, tetramers are partially converted to heterodimers.[380] One-dimensional[276] and two-dimensional peptide-mapping analyses[280,289] have indicated that the TW 240-kDa subunit is equivalent to avian α RBC or brain spectrin, while the TW 260-kDa β subunit is distinct from the 235- or 220-kDa β subunits of avian brain or RBC spectrin. The 260-kDa subunit is phosphorylated *in situ* by an endogenous brush border kinase.[381]

2. Protein Interactions

a. Calmodulin

The 240-kDa α subunit of TW 260/240 binds calmodulin in a Ca^{2+}-dependent manner when assayed by the gel overlay technique.[275,276] The functional significance of this interaction has not yet been determined.

b. Actin

TW 260/240 binds to f-actin in sedimentation studies,[275,380] and cross-links the f-actin solutions as apparent from increased low shear and high shear viscosity.[275,380] The binding of TW 260/240 to f-actin was optimal at low ionic strength (25 m*M* KCl) and a pH of 7.0, decreasing substantially at 150 m*M* salt or higher pH (7.5).[380] Skeletal muscle tropomyosin added at a 1:7 ratio to actin monomers had no effect on the binding of TW 260/240.[380] High-resolution microscopy of the TW 260/240-f-actin complex suggested that the ends of TW 260/240 actually wrap around the actin filament[381] rather than bind tangentially, as in the case of RBC and brain spectrin.

c. Ankyrin

While avian RBC spectrin binds to human erythrocyte spectrin-depleted-inverted vesicles (IOVs) with high affinity (K_D = 50 n*M*), TW 260/240 bound very weakly (K_D = 1000 n*M*).[382] The binding of RBC spectrin was inhibited by addition of exogenous ankyrin, while the weak binding of TW 260/240 was not. Furthermore, in solution, chicken RBC spectrin bound to RBC ankyrin with high affinity, while TW 260/240 did not bind. These findings suggested that TW 260/240 does not contain an ankyrin binding site.[382] Furthermore, no immunoreactive analog of ankyrin was detected in avian brush borders, leading to the suggestion that, while TW 260/240 cross-links the actin core bundles, it probably cannot link these core rootlets to membranes through an ankyrin-mediated interaction.

d. Protein 4.1

Coleman et al.[110] demonstrated that the binding of TW 260/240 to f-actin is not stimulated by human RBC protein 4.1. Furthermore, while isolated TW 240 and RBC β subunits did not bind to f-actin in the presence of 4.1, a hybrid complex of these subunits did bind. These results suggested that TW 260/240 does not contain a protein 4.1-binding site. Immunological characterization of avian brush border protein indicated a lack of a protein 4.1 analog, suggesting that TW 260/240 is probably not membrane-associated through a 4.1-mediated interaction.

Table 2
POTENTIAL AND KNOWN FUNCTIONS OF MEMBRANE-BOUND VS. CYTOPLASMIC MATRIX SPECTRINS

Membrane-associated spectrin	Cytoplasmic matrix spectrin
Stabilizes the membrane	Stabilizes the cytoskeletal matrix
Defines the membrane contour	Defines the compartmental shape
Controls lateral mobility of integral membrane proteins	Controls the translocation of vesicles
Regulates transbilayer movement of phospholipids (flip-flop)	Regulates the movement and placement of organelles

3. Location and Function

Glenney and Glenney[280] initially demonstrated with subunit-specific antibodies that TW 260/240 was not expressed in the earliest stages of development of avian intestine (<15 d). However, intestinal spectrin(240/235) was observed in the earliest stages of development by indirect immunofluorescence. Later in development (17 to 18 d), TW 260/240 is expressed and observed in the apical brush border regions of these cells, while intestinal spectrin(240/235) completely lined the membranes of these same cells.

Subsequent immunoelectron microscopy of chicken intestinal brush borders with the subunit-specific antibodies followed by a secondary ferritin-labeled antibody indicated that TW 260/240 represents the 5-nm wide filaments which cross-link core rootlets at all levels of the terminal web.[383] Therefore, TW 260/240 serves as a core rootlet-connecting fibril and also serves a stabilizing function within the terminal web. The TW 260/240 did not appear to link core rootlets to membranes, consistent with the lack of ankyrin- or protein 4.1-binding sites on TW 260/240. Interestingly, in mammalian brush border where the spectrin analog is of the 240/235 variety (no TW 260/240 is present),[289] Hirokawa et al.[384] noted that spectrin cross-links not only interconnect core rootlets, but also appear to connect core rootlets to the apical plasma membrane, to intermediate filaments, and to vesicles embedded in the terminal web. The mammalian intestinal spectrin(240/235) may well have ankyrin- and protein 4.1-binding sites which could be involved in membrane linkage. Therefore, in mammalian intestine the spectrin(240/235), in addition to stabilizing core rootlets, is probably also involved in the stability of membrane surfaces and in regulating the cytoplasmic trafficking of vesicles.

IV. CONCLUSION

There has been explosive growth in the spectrin literature since the discovery of nonerythroid spectrin.[9,10] We felt that this might be the last opportunity to write a truly comprehensive review covering both erythroid and nonerythroid spectrins and related molecules in a single (albeit lengthy) article. The timing also appeared right because the literature describing the existence, structure, and location of nonerythroid spectrin molecules has begun to give way to an understanding of function.

While descriptions of protein structure and location have their own intrinsic merit, their true value lies in the fact that they bring us a few steps closer to understanding function. Within this review, we have focused on erythrocyte spectrin, TW 260/240, and brain spectrin isoforms, each of which represents the archetype of a functional class of spectrins. In Table 2, we have listed potential and known functions of membrane-associated and cytoplasmic matrix spectrins. Erythrocyte spectrin is found on the cytoplasmic surface of the RBC plasma membrane, and it serves the four functions listed under membrane-associated spectrins in Table 2. TW 260/240,

in contrast, is found solely in the terminal web region of avian intestinal epithelial cells. It is a component of the cytoplasmic matrix, but does not appear to be membrane-associated. The suggested functions of a cytoplasmic matrix spectrin, which are listed in Table 2, include stabilizing the cytoskeletal matrix, defining compartmental shape, and controlling translocation of vesicles and organelles. It should be noted that the suggested roles of a cytoplasmic matrix spectrin parallel those of a membrane-associated spectrin, but are applicable to the three-dimensional space of the cytoplasm rather than the two-dimensional plane of the membrane. While a membrane-associated spectrin stabilizes the two-dimensional membrane fabric, the cytoplasmic matrix spectrin stabilizes the three-dimensional interdigitating cytoskeletal matrix. While the membrane-associated spectrins define membrane contour, the cytoplasmic matrix spectrins contribute to the shape of cellular compartments (e.g., the axon). Finally, the membrane-associated spectrins control lateral mobility of integral membrane proteins and transbilayer movement of phospholipids through the two-dimensional membrane, while the cytoplasmic matrix spectrin controls the translocation of vesicles and organelles within the three-dimensional cytoplasmic space. TW 260/240 falls into our classification of a cytoplasmic matrix spectrin, and the functions listed in Table 2 are consistent with electron microscopic images of TW 260/240 *in situ*[383] and its interactions with f-actin *in vitro*.[275,380] The four suggested functions of a cytoplasmic matrix spectrin reasonably fit TW 260/240, but remain to be proven.

The brain spectrin isoforms are archetypal examples of the most versatile class of spectrins, which fit both the membrane-associated and cytoplasmic matrix spectrin classifications. Both mammalian brain spectrin(240/235) and (240/235E) are found associated with the cytoplasmic surface of the plasma membrane and organelle membranes.[297] Therefore, we would predict that these brain spectrin isoforms should fit the functional roles listed under membrane-associated spectrins in Table 2. Indeed, there is already highly suggestive evidence that brain spectrin(240/235E) can control the lateral mobility of $N\text{-}CAM_{180}$ within the neuronal plasma membrane.[366,367] In addition, both brain spectrin isoforms are components of the cytoplasmic matrix and appear to function as described in Table 2 for this classification of spectrins. Both spectrin isoforms cross-link actin filaments, microtubules, and neurofilaments and therefore can be reasonably expected to stabilize the cytoskeletal matrix and define the shape of axons and dendrites.[297] Furthermore, there is indirect but highly suggestive evidence that brain spectrin(240/235) controls the translocation of synaptic vesicles and regulates the axonal transport of organelles and cytoskeletal structures. While much of Table 2 remains to be proven, we believe that its value is in its predictive power for the function of other less well-studied nonerythroid spectrin molecules of known intercellular location.

The growth of our knowledge concerning the structure and location of nonerythroid spectrin molecules since their discovery in 1981 has been astonishing. We expect that over the next 5 to 10 years our knowledge of the function of the nonerythroid spectrin molecules will approach our current detailed understanding of the erythrocyte spectrin membrane skeleton.

ADDENDUM

After completion of this review, we became aware of three noteworthy recent observations. Gardner and Bennett have demonstrated that a calmodulin-binding protein, they named adducin, has the capacity to bind to spectrin-actin complexes.[387] The binding of adducin to spectrin-actin complexes promotes assembly of additional spectrin molecules onto actin filaments, a process which is inhibited by calmodulin at physiological concentrations of Ca^{2+}.

In preliminary reports, Harris and Morrow mapped the Ca^{2+}-dependent calmodulin-binding site to a position toward the center of the a subunit of brain spectrin,[388] and Conboy et al.[389] demonstrated that proteins 4.1a and 4.1b are generated by a mechanism that includes alternative splicing of the primary 4.1 RNA transcript.

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