J. Membrane Biol. 51, 101–131 (1979)

Topical Review

Spectrin: Present Status of a Putative Cyto-Skeletal Protein of the Red Cell Membrane

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Received 1 October 1979

Introduction

All species of red blood cells that have been examined have two high molecular weight polypeptide chains which are easily extracted in water-soluble form by exposing the membranes to low ionic strength buffers. The eluted polypeptides, named spectrin [43], comprise approximately 20–30% of the total membrane protein. Spectrin is soluble in aqueous buffers, but its molecular size and shape under these conditions are still in question. Spectrin is clearly located along the inner surface of the red cell membrane, but it is not certain how and by what means it attaches to this surface. It has been suggested that spectrin binds to specific acceptor proteins, or possibly to a complex of acceptor proteins, but other evidence suggests that spectrin might bind to phospholipids of the membrane or to a combination of both lipids and specific acceptor proteins. It is also conceivable that spectrin is not tightly bound to the inner surface of the membrane at all, but is present instead as a two-dimensional network which interacts loosely with the overlying lipid bilayer.

The presence of such abundant amounts of this high molecular weight protein has stimulated many to explore the possibility that this molecule might be involved in determining the shape of red blood cells and possibly also in stabilizing the lipid bilayer. More recently it has been suggested that spectrin may contribute to or be responsible for some of the lipid asymmetry of the red cell membrane. Other experiments are consistent with the idea that spectrin may influence the distribution of the integral

0022-2631/79/0051-0101 $06.20

membrane proteins, parts of which are imbedded within the lipid matrix of the bilayer. Preliminary investigations into the possible relationships between spectrin and these integral membrane proteins indicated that spectrin might bind to the cytoplasmic segments of these molecules, but the results of more recent experiments question whether spectrin binds directly to these proteins.

Since the subunits of spectrin are in the myosin size-class and spectrin is often extracted in company with an erythrocyte form of actin, it had been suggested that spectrin and actin might form an acto myosin-like complex. The idea that such a contractile apparatus might influence red cell shape seemed attractive, but there are now reasons for considering such a model to be unlikely.

Early electron microscopic studies using conventional thin section techniques revealed the presence of fibrillar material along the inner surface of red cell ghosts, consistent with the presence of a protein network; further support for such a network has come from recent scanning electron microscopic studies of ghost membranes partially extracted with detergents. The integral membrane proteins and some of the membrane lipids can be selectively extracted with Triton X-100, leaving behind a web-like structure which seems to correspond to a spectrin-actin network. One of the problems in evaluating these findings is that isolated spectrin and actin have never been shown to reassemble in such an arrangement *in vitro*.

At least two secondary modifications of the spectrin molecule have been described, and each has been implicated one way or another in its function. One of the spectrin subunits is phosphorylated, and some preliminary experiments suggest that the phosphorylation-dephosphorylation reactions of spectrin may influence the interactions of spectrin with other proteins. Spectrin can also undergo a γ-glutamyl-lysine cross-linking reaction which is catalyzed by a calcium-activated transglutaminase. The activity of this enzyme is influenced by calcium which is in turn influenced by ATP levels in the cells. On the basis of these relationships an intriguing hypothesis has been created in which ATP levels could cause irreversible cross-linking of the spectrin network. Such a process might play some role in removing old red cells from the circulation.

Many investigators have searched for special molecular features of the spectrin molecule which could explain its capacity to interact with cell membranes and perhaps explain its contributions to lipid stability and red cell shape. A search has been made for the presence of γ-carboxyl

glutamic acid residues in spectrin molecules, but so far results have been negative. Spectrin has a high content of glutamic residues, most of which are probably in the acidic form, and it would have been reasonable for red cells to use the γ-carboxylation mechanism to produce spectrin molecules able to associate with acidic phospholipids by calcium bridges, as has been postulated for prothrombin binding.

Others have wondered whether spectrin is made up of smaller subunits which are secondarily linked together to form high molecular weight chains. There were earlier reports that the high molecular weight forms of spectrin represented noncovalently linked aggregation states of smaller peptides, but this interesting idea seems not to be true. One of the problems in speculating as to the significance of any unique molecular feature of spectrin is that we are not sure whether spectrin exists as a flexible rod or whether it has the capacity to form more complex forms. Some recent experimental data suggests that there may be transition states from a rod-like form of spectrin to a more complex pseudo-globular form. Isolated spectrin molecules also exist in a number of different high molecular weight aggregation states, depending upon how spectrin is prepared or how the red cells were pretreated before osmotic lysis. ATP levels seem to be influential in this regard.

Most students of spectrin recognize a number of questions which remain to be answered:

i) Is spectrin really composed of two unique high molecular weight polypeptide chains which are organized as flexible rods?

ii) Are spectrin molecules isolated by low ionic strength buffers in the native, undenatured state? The ease of extracting spectrin from red cell membranes by this approach has resulted in its almost universal application to studies of spectrin. Since physical studies of this molecule have been so inconsistent from laboratory to laboratory, many have wondered whether spectrin is partially denatured during the extraction procedure. From the above a third nagging question follows.

iii) What is the most physiological way to study the functions of the spectrin molecule?

Purified spectrin binds to inside-out vesicles of ghost membranes and it also binds to liposomes prepared from acidic phospholipids. The results of studies of the binding of spectrin to inside-out vesicles suggest that spectrin may bind to a specific acceptor protein which is near or part of a multiprotein complex. However, the binding of spectrin to either red cell vesicles or to liposomes may not approximate the conditions that spectrin encounters in the intact erythrocyte. Spectrin may

be associated with actin *in vivo*, but so far no convincing models for a spectrin-actin network have been formulated, nor have "functional" complexes between spectrin and other proteins been reconstituted *in vitro*.

A number of detailed reviews of the spectrin literature have appeared in recent years [29, 37]; since these cover specific areas of spectrin research in great detail, this review will attempt to concentrate on data and ideas which have been generated over the most recent past.

Properties of Isolated Spectrin

The human erythrocyte spectrin molecule is a tetramer composed of two copies of two different polypeptide chains with an aggregate mol wt of approximately 900,000. Spectrin is usually isolated after incubating red cell ghost membranes in dilute buffers containing EDTA at 37° for short periods [40], and under these conditions the predominant form is a dimer composed of one copy of each chain. If the extraction procedure is carried out at 4° instead of 37° a large fraction of the solubilized spectrin is in the tetramer form [56]. The individual polypeptide chains of spectrin have been referred to as Bands 1 and 2 on the basis of their mobilities on SDS gels [17]; these will be referred to here as the α and β subunits, respectively. The appearance of spectrin on SDS gels is shown in Fig 1.

The α-chain has an apparent mol wt of 240,000 daltons; the β subunits is slightly smaller (mol wt approximately 220,000 daltons), but, as will be pointed out below, the β-chain is not simply a smaller version of the α-chain. The amino acid compositions of the two chains is extremely similar (Table 1), but the peptide maps generated by a variety of different ways indicate that the two chains have significant differences in their primary structures [39, 74][1]. The mammoth size of both polypeptide chains has greatly hampered attempts to purify each of the individual polypeptides, and attempts at conventional ion exchange chromatography and preparative isoelectric focusing have been relatively ineffectual; the only way to isolate spectrin free of other proteins involves the use of low resolution gel filtration techniques in different salt solutions.

The purification of spectrin has also been hampered by the lack of a suitable assay, but since the "functions" of spectrin are not well

[1] D. Speicher, J. Morrow, W. Knowles & V.T. Marchesi (*manuscript in preparation*).

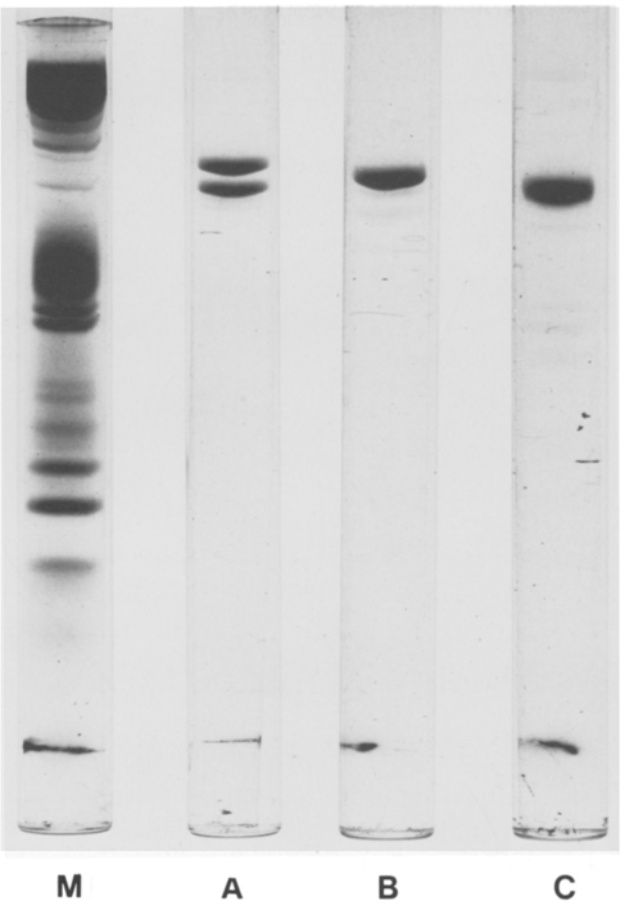

Fig. 1. SDS acrylamide gel electrophoresis of red cell ghost membranes (*M*), spectrin (*A*), purified α subunit (*B*), and purified β subunit (*C*)

understood it is difficult to devise appropriate *in vitro* assays. For this reason we cannot be sure that the two high molecular weight polypeptide chains represent the complete, native molecule. It would not come as a great surprise if one or several small molecular weight peptides were found to be part of the functional molecule, analogous to the light chains of myosin. Although there is yet no reason to suspect that this will prove to be the case, the methods used for spectrin isolation by almost all investigators were designed to selectively isolate the two high molecular weight polypeptides free from all smaller components. Thus potential cofactors or modifying peptides which might not be released from the membrane under the same conditions as the higher molecular

Table 1. Amino acid composition of human spectrin subunits (in mole %)

Amino acid	Band 1	Band 2	Spectrin
Asp	11.4	10.5	10.8
Thr	3.8	3.8	3.9
Ser	5.1	5.1	5.2
Glu	20.8	20.1	20.1
Pro	1.7	2.3	2.1
Gly	4.4	5.0	4.7
Ala	9.2	9.4	9.3
Val	4.8	5.1	4.8
Met	0.9	1.1	1.1
Ile	3.4	3.3	3.5
Leu	14.0	14.5	14.2
Tyr	2.2	1.9	2.1
Phe	3.5	3.5	3.4
His	2.9	3.1	3.0
Lys	7.6	6.6	7.1
Arg	5.8	6.7	6.4

weight polypeptides would be systematically lost during the isolation procedure.

Attempts to determine the homogeneity of the spectrin polypeptide chains by isoelectric focusing in the presence of urea or nonionic detergents have met with limited success. Several investigators have noted that the spectrin polypeptides focus as extremely broad bands over wide pH ranges, and, on the basis of these results, have suggested that spectrin is composed of a large number of similar but chemically distinct polypeptide chains [7]. This approach has recently been re-examined using spectrin purified in different ways, and it has been found that the results obtained from isoelectric focusing studies of spectrin are critically dependent upon the absolute amounts of spectrin applied to the gels. If low amounts of protein are applied, relatively narrow bands of spectrin are produced which focus in the pH range of 5.0 to 5.2 [24].

Another indication that spectrin may be composed of multiple polypeptide chains comes from attempts to identify the N-terminal amino acids of the spectrin polypeptides using conventional techniques for end group analysis [18, 31, 13]. Three different research groups have generated a number of different PTH derivatives, consistent with the presence of multiple polypeptide chains. Although the simplest interpretation of these findings is polypeptide heterogeneity, many people question the validity of these findings. None of the studies have produced quantitative

recoveries of the N-terminal residues, and although great pains have been taken to exclude the presence of contaminating polypeptides, the lack of reliable methods for separating spectrin from other polypeptides (as described above) complicates these studies. Multiple N-terminal amino acids could also be generated from otherwise homogeneous spectrin polypeptides if one or both of the polypeptides was contaminated by some partially degraded forms. It is interesting that a recent study by Anderson shows that the terminal ends of the spectrin subunits are more sensitive to proteolytic cleavage than internal sites [2].

Recently Hudson and Ralston have attempted to quantitate the number of N-terminal residues of the spectrin subunits by using a transamination reaction, and they found only one amino terminal residue per spectrin monomer [25].

It is likely that the question of spectrin's homogeneity will only be resolved when peptide fractionation and amino acid sequencing studies are carried out, but on the basis of the evidence at hand it seems unlikely that these mammoth polypeptide subunits of spectrin are really heterogeneous. Preliminary studies in this laboratory indicate that the α and β subunits of spectrin are both homogeneous polypeptides, and are also distinctly different from each other[2]. Thus on these grounds and on the basis of other evidence described below most investigators have assumed that the spectrin subunits are homogeneous entities, and no effort has been made to isolate and study subfractions of spectrin molecules. Of course if this presumption proves to be incorrect, the task of deciphering the structure and functional components of the spectrin subunits will be even more formidable.

The Spectrin Tetramer Appears to be a Flexible Rod

The early studies on the structure of spectrin, which now seem extremely primitive in retrospect, all indicated that spectrin was some kind of a "fibrous protein". This impression was gained primarily from negative staining of water-soluble extracts of red cell ghosts which were composed largely but not exclusively of the high molecular weight subunits of spectrin. This material often appeared as filamentous structures of irregular periodicity and indeterminant length [64]. Since it seemed likely that spectrin was sensitive to high concentrations of salt, particularly the heavy metal salts used for negative staining, most investigators tended

[2] *See* footnote 1, p. 104.

to attribute these findings to artifacts of the negative staining technique. When more conventional physical studies of spectrin molecules were undertaken, the results were even more confusing, since studies of ostensibly similar spectrin preparations by sedimentation velocity and sedimentation equilibrium analysis produced an assortment of conflicting results.

A careful study of the molecular sizes of the spectrin subunits in the presence of the detergent deoxycholate indicated that the spectrin dimers had apparent molecular weights in the 500,000 range. However, fractions of spectrin thought to be dimers had sedimentation coefficients which were almost identical to spectrin monomers. On the basis of this striking discrepancy between sedimentation coefficients and molecular weight, it was suggested that spectrin monomers were elongated rods approximately 800 Å in length and dimers were similarly shaped rods 1,600 Å in length [54]. The findings summarized in Table 2 illustrate the great variations obtained from physical studies of spectrin in different laboratories.

Up until recently most electron microscopic studies of isolated spectrin provided little help in choosing between the different physical models. Although conventional negative staining techniques have been largely abandoned in recent years, many investigators have attempted to determine the size and shape of spectrin molecules using heavy metal shadow techniques which have proved so successful in outlining molecules of this type in the past. For reasons which are still unclear this approach also failed to yield satisfactory results until Branton and co-workers modified the procedures used to prepare spectrin for shadowing. The initial results from this laboratory suggested that spectrin dimers (α, β) were composed of three amorphous pseudo-globular forms which were arranged in a linear array, somewhat reminiscent of the shadowed preparations of fibrinogen. These results were unsatisfactory for many reasons, among them the fact that these findings did not agree with most of the results from physical studies summarized in Table 2. These investigators persevered and eventually discovered that an entirely different morphology of the spectrin molecule could be obtained if spectrin was suspended in 70% glycerol prior to the shadowing procedure [60]. Spectrin isolated from red cell ghost membranes by suspending the membranes in dilute buffers containing EDTA at 37° is largely composed of a dimer form with a mol wt of approximately 450,000, and this form appears to be a flexible rod of approximately 1,000 Å in length. When spectrin is prepared by extracting ghost membranes at low temperatures, for

Table 2. Physical studies of isolated spectrin

Schecter, Sharp, Reynolds Tanford, 1976 [57]	$S_{20}W$ of monomer 5.5 S in deoxycholate $S_{20}W$ of dimer 6.0 S Mol wt of dimer by sedimentation equilibrium ~500,000 Assuming monomer and dimer are both cylindrical rods, dimensions calculated to be 24Å × 830Å (monomer) 24Å × 1660Å (dimer)
Ralston, 1976 [56]	$S_{20}W$ of dimers and tetramers found to be 8.5 S and 12 S Mol wt of tetramer ~1,000,000 Hydrodynamic properties suggest a highly expanded but basically symmetrical molecule of stokes radium 200Å
Kam, Josephs, Eisenberg Gratzer, 1977 [27]	$S_{20}W$ of dimer 9.5 S Mol wt of dimer ~500,000 Axial Ratio ~2–10 Radium of gyration ~80Å (upper limit) Electron microscopy of negatively stained preparations suggest dimer is a compact, slightly elongated unit tetramer probably two parallel dimers
Shotten, Burke & Branton, 1978 [61]	Electron micrographs of low angle shadowed dimers show flexible rods 1,000 Å long; 12 S tetramers appear as 2,000Å rods
Elgsaeter, 1978 [14]	Light scattering measurements of dimers indicate a radius of gyration 400Å, suggesting that isolated spectrin dimers are flexible rods the lengths of which are in the 760Å to 1,400Å range

The enormous variability in these results cannot be attributed simply to observer error, but more likely is due to subtle differences in spectrin preparations, depending upon the age and state of the donor red cells and the conditions used for isolation, purification, and analysis.

reasons to be described below, a substantial fraction of the spectrin is in the tetrameric form with a mol wt of approximately 900,000. This tetrameric unit also appears as a flexible rod and has an approximate length of 2,000 Å, consistent with the tetramer form being composed of two end-to-end associated dimers. Examples of such preparations are shown in Fig. 2.

These most recent findings agree with our preconceived notions as to what the spectrin molecule should look like, but it is important to realize that this data is obtained from shadowed material prepared by suspending spectrin in high concentrations of glycerol. Although this treatment has been employed to great advantage in the study of other

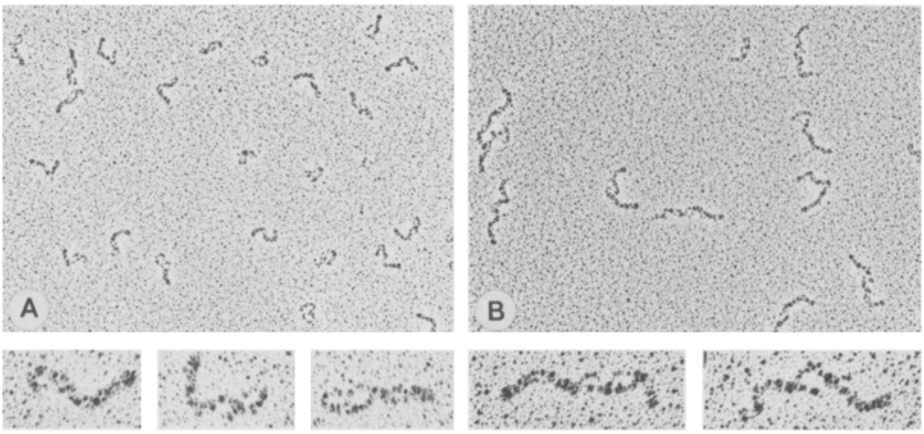

Fig. 2. Spectrin dimer (*A*) and tetramer (*B*) viewed by low-angle rotary shadowing electron microscopy. Large panels, ×60,000; small panels, ×108,000. From the work of Tyler, Shotten and Branton [62, 66]

macromolecules, and there is no reason to suspect artifactual changes in the morphology of the molecules, it will be important to confirm this structure using other techniques and on preparations prepared in different ways. Some indication that this flexible rod model is probably close to reality has been provided by some recent light scattering experiments [14] and by the results of controlled proteolytic digestion experiments [3].

The relationship between the dimeric form of spectrin which is the predominant species when spectrin is prepared at 37° and the tetrameric form which is prevalent when spectrin is prepared in the cold has recently been clarified by studies by Gratzer and co-workers [67]. These investigators have discovered that the dimer and tetrameric states of spectrin are in an equilibrium which has a unique temperature requirement in order to jump from the dimer to the tetramer form. This transition takes place at approximately 28 °C and results in tetramers which are unstable at higher temperatures in the isolated form. Since the tetramer is the largest unit of spectrin regularly produced *in vitro*, it is likely to be present as such in the intact red cell, and we assume its instability at 37° is an *in vitro* artifact. Perhaps, as mentioned earlier, spectrin may exist in the company of stabilizing peptides or other cofactors which have been lost during the isolation and purification steps. It is also possible that spectrin prepared by low ionic strength extraction techniques is partially denatured, and this could account for its relative heat lability

[3] *See* footnote 1, p. 104.

since the conformation of spectrin can be easily disrupted by conventional denaturing agents [24, 57]. However, if soluble spectrin is modified during extraction the changes are certainly not extensive since spectrin prepared in a variety of ways has a large amount of tertiary structure when analyzed by circular dichroism [24, 55].

Conformation of Spectrin

As described above, the most appealing model for spectrin in solution is that of a flexible rod, either 1,000 Å long for the dimer or 2,000 Å long for the tetramer. The clearest evidence in support of this model has been obtained from rotary shadowing studies and conventional transmission electron microscopy. Supporting evidence has also been provided by recent light-scattering data and the model is consistent with the earlier electron microscopic studies of isolated spectrin by negative staining. It is interesting that many of the early studies of the ultrastructure of ghost membranes by thin sectioning techniques also suggested the presence of fibrillar structures which were presumed to be spectrin [42] and, in retrospect, it seems reasonable that these fibrils could have been created by side-to-side association of the flexible rod-like molecules illustrated in Fig. 2. The same interpretation can be applied to the thick cords of material seen in the reticular network which remains with ghost membranes after the integral proteins and most of the lipid is extracted away by Triton X-100 [22].

Soluble spectrin has a substantial amount of α-helical conformation as determined by circular dichroism [24, 55]. Similar spectra are obtained from spectrin isolated by a variety of different treatments, including spectrin which has been purified in SDS [24]. Purification of spectrin in SDS is the simplest way to isolate spectrin polypeptides free from contaminating elements, and it is the only available method to isolate substantial amounts of the individual spectrin subunits [33]. Large amounts of the spectrin subunits have been isolated by applying recycling technology to SDS gel filtration[4]. The isolated subunits lose most of their α-helical conformation in the presence of SDS, but their helicity returns after the SDS is removed by dialysis [24]. These spectra are shown in Fig. 3. This reversal seemed surprising when it was first observed, but it is now evident that denatured spectrin is able to refold rapidly and with great fidelity[5]. It is interesting that both spectrin subunits can refold into helical segments independently of each other.

[4] *See* footnote 1, p. 104.

[5] W. Knowles, D. Speicher, J. Morrow, V.T. Marchesi (*manuscript in preparation*).

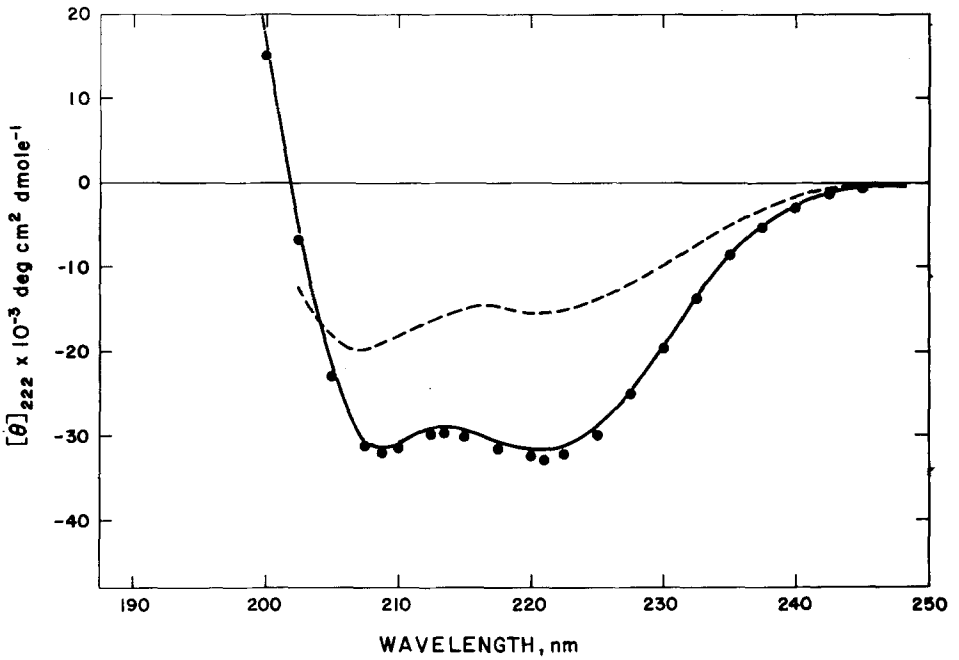

Fig. 3. Circular dichroism spectra of spectrin. (——) is spectrin isolated in 25 mM Tris·HCl, 150 mM NaCl, pH 9.0, and dialyzed into 100 mM Na-phosphate buffer, pH 7.2 (0.211 mg/ml). (●●●) is spectrin isolated in 0.1% SDS buffer (10 mM Tris·HCl, pH 9.5, 0.5 mM Me, 0.1% SDS). After removal of SDS, the spectrin is dialyzed into 100 mM Na-phosphate buffer, pH 7.2 (0.197 mg/ml). (---) is spectrin in the same 0.1% SDS buffer (0.23 mg/ml). Spectra were results of three recordings on three different preparations. Taken from Hsu *et al.* [24]

Isolated α and β chains of spectrin have been subjected to controlled proteolytic digestion and to various forms of chemical cleavage to determine how similar they are to each other. Although no amino acid sequence data are available yet it appears on the basis of limited proteolytic cleavage experiments that both chains are similar in overall composition, but the β chain is not simply a shorter version of the α chain[6]. As will be described below, the β chain is the only subunit of spectrin which is phosphorylated *in vivo* and it is the only subunit which can bind to inside-out ghost vesicles.

Post-Translational Modifications of the Spectrin Molecule

It has been known for some time that the β subunit of the spectrin molecule can be phosphorylated when red cell ghost membranes are

[6] *See* footnote 1, p. 104.

incubated with ATP under appropriate conditions, and more recently it has been shown that this subunit is also phosphorylated in intact red blood cells [70]. Although many have speculated that the phosphorylation of this membrane protein must play some critical role in its function, arguing by analogy with other membrane-related phosphorylation reactions, the evidence linking spectrin phosphorylation with any of its putative functional roles is still fairly indirect. Birchmeier and Singer have shown that the removal of phosphate groups on the spectrin molecule by incubating red cell ghost membranes with phosphatases results in striking changes in the shape and deformability of the ghost membranes [8]. The phosphorylation state of spectrin may also play some role in mediating interactions between spectrin and actin, but the results from different labs are in conflict. Spectrin and actin form gel-like structures *in vitro* when spectrin is appropriately phosphorylated [50], but these observations are complicated by the fact that crude preparations of spectrin were used in this study. Cohen, Jackson, and Branton have shown that purified preparations of spectrin do not show the same reactivity [11]. Similarly, others have recently shown that the spectrin tetramer can bind to the F-form of actin but not the G-form, regardless of the degree of phosphorylation [9].

There is no unanimous consensus as to the number of phosporylation sites per spectrin molecule. The number of sites seems to vary with the types of red cell ghost membranes and the conditions under which phosphorylation is carried out. Several recent studies suggest that the phosphopeptides of the β subunit are located close to one of the terminal ends of the polypeptide chain, since the phorphorylated segments can be removed by mild proteolytic digestion which appears to cleave only a small peptide from either the amino or the carboxyl-terminal end of the molecule [2]. Phosphorylated peptides have also been isolated from the β subunit of spectrin by proteolytic cleavage [71]. The initital enthusiasm for following up studies of spectrin phosphorylation was somewhat dampened by the realization that spectrin phosphorylation was not cyclic nucleotide dependent. However, since human red cells contain little if any adenyl cyclase it is not surprising that this modulating mechanism does not operate in these cells, and it does not really diminish the potential physiologic significance of spectrin phosphorylation. Studies on the number and types of kinases which may be involved with spectrin phosphorylation suggest that the regulation of phosphorylation may reside at this level [16]. It has also been shown recently that the state of spectrin's phosphorylation does not seem to influence the dimer-tet-

ramer conversion [67], nor does it seem to influence the binding of spectrin to the inside surface of ghost membranes. However, another membrane protein, Band 2.1, is phosphorylated in intact red cells [5], and there are reasons for implicating this protein in the binding of spectrin to membranes, as described below.

Spectrin does not appear to be glycosylated, but no really systematic studies of this question have been carried out beyond the preliminary studies of crude spectrin preparations carried out some years ago [43]. Since most cytoplasmic proteins are not glycosylated, there has been little incentive for other investigators to substantiate this finding, and it seems unlikely that significant amounts of sugar will be found covalently attached to spectrin molecules obtained from normal red cells. However, it remains an interesting possibility that spectrins isolated from red blood cells from patients with diabetes mellitus or under other conditions of hyperglycemia may have glycosylation of their spectrin chains as a result of the high levels of glucose forming covalent attachments to exposed amino groups, similar to the mechanism proposed for the glycosylation of hemoglobin [10].

Several investigators have suggested that significant amounts of isopeptide bonds are present in proteins of red cell membranes as well as other mammalian cell membranes. This idea was originally stimulated by the finding of glutamyl-lysine dipeptides in proteolytic digests of crude membrane protein preparations. Recently it has been found that there is a calcium-activated transglutaminase in red cells which is able to form glutamyl-lysine bonds between polypeptide chains of different subunits of spectrin [35]. As a result of this reaction high molecular weight forms of spectrin have been produced following incubation of intact red cells with calcium and calcium ionophores. High levels of calcium can also induce the same reaction in isolated red cell ghost membranes [1]. Transglutaminase-catalyzed bonds can also be formed between spectrin molecules and other membrane proteins, such as Band 3 and Band 4.1 [35]. These results suggest that the spectrin subunits exist in the membrane in close proximity to these membrane proteins and are consistent with some recent data on the binding of spectrin to red cell membranes to be described below. These results also confirm data obtained with chemical cross-linking agents [68] and results obtained following acid-induced disulfide bond formation in red cell ghost membranes [34].

Endogenous transglutaminase activity in red cells has proved to be a useful tool to probe nearest-neighbor relationships between membrane

proteins, but its physiological significance still remains undefined. Half-maximal activity of the enzyme required 0.3 mM calcium, which is significantly higher than the free calcium concentration found in the cytoplasm of red cells. Lorand has offered the provocative suggestion that this calcium-activated cross-linking reaction may be one of the mechanisms built into red cells to promote the removal of old red cells from the circulation when the metabolic machinery of the cells is no longer capable of generating appropriate levels of ATP. He has hypothesized that old red cells lose their capacity to generate ATP, probably by loss or denaturation of appropriate glycolytic enzymes, and once ATP levels drop below a certain range, it is presumed that red cells lose their capacity to pump calcium out of the cytoplasm. If calcium accumulates to high enough levels, it is able to activate the transglutaminase which in turn cross-links and thereby aggregates neighboring spectrin polypeptides. It is assumed that highly cross-linked spectrin will render red cell membranes less deformable and thereby less capable of navigating through the microcirculation. Teleologically the whole operation makes sense: if ATP levels in red cells drop below a certain level, the cells can no longer operate their pumps, they will probably become defective oxygen and CO_2 carriers, and they should be removed to make way for a new population. By gradually stiffening their external shells they become more susceptible to the physiological entrapment mechanisms of the reticulo-endothelial system and are thus likely to be removed in the spleen rather than collecting in the microcirculation of more critical sites.

Spectrin Does Not Have γ-Carboxyl Glutamic Acid Residues

Isolated spectrin molecules do not seem to have detectable amounts of γ-carboxyl glutamic acid residues. Although no systematic analysis of this question has yet been published, several laboratories have attempted to detect such residues without success[7, 8]. Since spectrin is easily extracted from red cell ghost membranes which have been suspended in low ionic strength media containing EDTA, it has been assumed that divalent cations might play some role in the attachment of spectrin to the inner surface of the red cell membrane. This idea was reinforced by observations suggesting that calcium had a specific

[7] C.J. Hsu & V.T. Marchesi (*unpublished observations*).

[8] S.E. Lux (*personal communication*).

capacity to induce the aggregation of isolated spectrin molecules, although the levels of calcium used were much higher than the values of free calcium ever encountered in the cytoplasm of normal red cells. The early attempts to explore the relationship between calcium and spectrin polymerization were hampered by the fact that most spectrin preparations were contaminated with significant amounts of actin-like material and possibly other unidentified proteins. Since calcium is able to enhance actin polymerization, some of the initial attempts to follow calcium-induced changes in the state of spectrin were undoubtedly complicated by the effects of calcium on contaminating elements. The subsequent realization that calcium also activated a transglutaminase which is able to covalently link spectrins also added to the confusion. A calcium-activated ATPase activity has been reported in spectrin preparations [30], but the levels of activity are extremely low, and the possibility that this ATPase is due to a contaminating enzyme cannot be ruled out.

The search for the presence of γ-carboxyl glutamic acid residues in spectrin was stimulated by the discovery that this unusual amino acid modification is a characteristic feature of some of the plasma proteins involved in blood clotting. It is now known that Vitamin K stimulates a carboxylation reaction through which glutamic acid residues are modified by the addition of a second carboxyl group at the gamma position [12]. The presence of this double carboxyl group seems to enhance the capacity of these proteins to bind to phospholipids in the presence of calcium. Although the precise mechanism by which calcium and the γ-carboxylated residues facilitate binding to phospholipids is not settled, it seems likely that calcium serves as a cationic bridge between the carboxyl groups of the plasma proteins and the anionic groups of the phospholipids. These mechanisms proposed for binding of plasma proteins to phospholipids during blood clotting have naturally stimulated membranologists to determine whether similar mechanisms can operate in plasma membranes. Since significant amounts of spectrin bind to the inner surface of the human red cell membrane, it seems likely that if this mechanism is important in lipid-protein interactions in cell membranes it would certainly operate here. So far, as mentioned above, preliminary results have been negative, and they are consistent with recent attempts to study the binding of spectrin back to the inner surfaces of red cell membranes using inside-out vesicles. Purified spectrin binds to inside-out vesicle membranes and, as described below, neither calcium nor magnesium is essential for binding; the only critical requirement is that a minimum salt concentration be present in the incubation media.

How is Spectrin Attached to the Membrane?

As mentioned above, spectrin is clearly associated with the inner surface of the red cell ghost membrane. This localization was first demonstrated by applying ferritin-conjugated anti-spectrin antibodies to red cell ghost membranes [46], and many subsequent studies employing radiolabeling techniques coupled with differential enzymatic digestion have shown quite clearly that spectrin molecules are not exposed to the external surfaces of intact red blood cells [41]. Most recently ferritin-conjugated antibodies have been used to confirm the submembranous localization of spectrin in intact human red blood cells using frozen sections of glutaraldehyde-fixed erythrocytes [73]. An example of this is shown in Fig. 4.

Efforts to determine where and how spectrin binds to the inner surface of the red cell membrane have produced three distinct hypotheses:

I. Spectrin binds to specific protein receptor molecules

II. Spectrin binds directly to lipids

III. Spectrin is part of a spectrin-actin cytoskeletal network.

The most persuasive evidence that spectrin binds to specific acceptor proteins attached to the inner surface of the red cell ghost membrane has been provided by studies of the binding of spectrin to inverted red cell ghost membranes (inside-out vesicles) [4][9]. Spectrin binds to sites with reasonably high affinity ($Ka \sim 10^7$) which are located exclusively on the inner surface of the ghost membrane. The binding of spectrin to the inside-out vesicles requires a minimal concentration of monovalent salt, but neither magnesium nor calcium ions are required for maximum binding, and the amount of spectrin which binds to the vesicles is roughly equivalent to that amount of spectrin known to be present on the inner surface of the ghost membrane. The binding of spectrin to the inside-out vesicles does not require the presence of any other proteins (actin is not required) and SDS-purified β chains of spectrin can bind to the inner surface of the ghost membrane and can compete with intact native spectrin for the same binding sites[10]. SDS-purified α chains do not bind to inside-out vesicles, and they are not able to compete with intact spectrin molecules.

The first significant clue that spectrin binds to specific acceptor proteins on the inside-out vesicles came from experiments showing that

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[9] D. Litman, C.J. Hsu & V.T. Marchesi (*manuscript submitted*).

[10] *Ibid.*

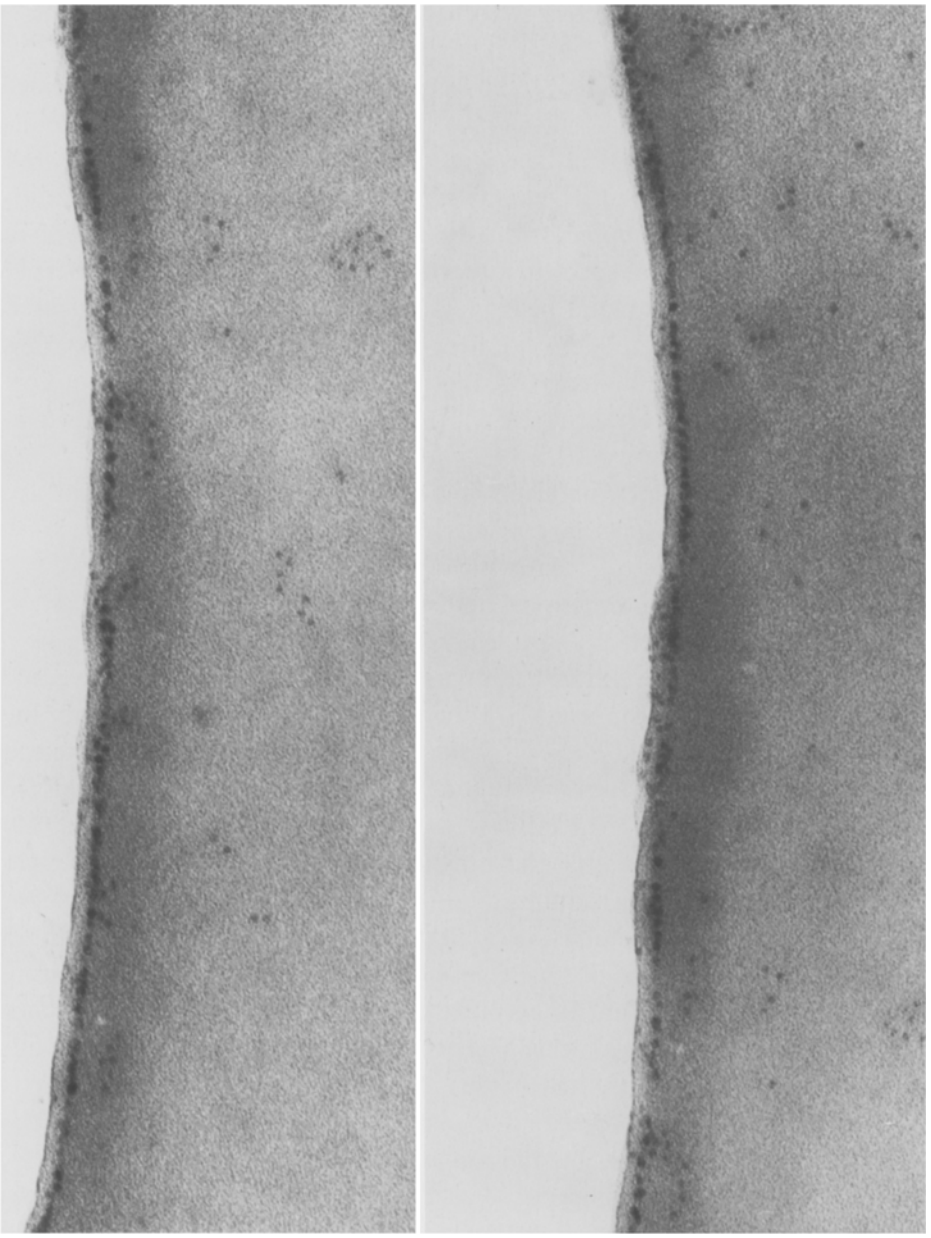

Fig. 4. Transmission electron micrograph of a frozen section of an intact human erythrocyte incubated with ferritin-conjugated anti-spectrin antibodies. The section was photographed without any heavy-metal staining. $\times 200{,}000$. Taken from Ziparo *et al.* [73]

mild proteolytic digestion or treatment of the vesicles with protein denaturing agents both modified the capacity of inside-out vesicles to bind spectrin. A detailed analysis of the peptide fragments released from membranes by chymotrypsin revealed that a 72,000-dalton peptide was released which was able to block the binding of spectrin to undigested ghost vesicles [3]. Subsequent studies have shown that this 72,000-dalton peptide is a proteolytic cleavage product of Band 2.1, a polypeptide with an apparent mol wt in the 200,000-dalton range. Detailed peptide mapping of Band 2.1 and the 72,000-dalton peptide confirm that the latter is derived from the former [5, 36]. The conclusion drawn from these studies is that Band 2.1, which has been named ankyrin [5], has the capacity to bind spectrin when the latter is incubated with inverted ghost vesicle membranes, and it is likely that this protein is one of the principal acceptor proteins for spectrin. As mentioned earlier, ankyrin is phosphorylated in red cells [5], and it is possible that the changes in red cell deformability following phosphatase treatment reported by Birchmeier and Singer [8] may be due to dephosphorylation of the ankyrin molecule rather than to the removal of phosphate from spectrin.

Specific antibodies against membrane proteins have also been used to identify the nature and the location of the spectrin binding sites. Antibodies directed against the cytoplasmic segment of Band 3 or those directed against a mixture of Bands 4.1–4.2 both inhibit the binding of spectrin to inside-out vesicles[11]. In contrast, comparable amounts of antibody directed against the cytoplasmic portion of glycophorin A bind to inside-out ghost vesicles but have no effect on spectrin binding. However, the relationship between Band 3 and spectrin binding is not a simple one, since the cytoplasmic domain of Band 3 does not bind to spectrin *in vitro* nor does it compete for binding sites on inside-out vesicles. These latter experiments suggest that the Band 3 molecules may be near or possibly part of a complex that is involved with spectrin binding to the membrane, but Band 3 itself probably does not contain specific binding sites for spectrin.

Similarly, the relationship between Bands 4.1–4.2 and spectrin binding is also not straightforward. A complex composed of Bands 4.1–4.2 can be isolated from red cell ghost membranes with detergents[12], and this complex is capable of associating with spectrin *in vitro*. Other evidence suggesting that spectrin and Band 4.1 may be close to each other in

[11] *See* footnote 9, p. 104.

[12] C.J. Hsu, D. Litman & V.T. Marchesi (*manuscript in preparation*).

the membrane comes from chemical cross-linking experiments and the results of endogenous transglutaminase activity [35]. However, the interpretation of chemical cross-linking experiments is not simple as Peters and Richards have recently pointed out [49], since many complexes between different membrane proteins are formed, and there is no way to determine the quantitative significance of any particular set of linked partners.

Another way to identify noncovalent complexes between membrane proteins has been to isolate complexes in the presence of non-ionic detergents. Steck and Yu [72] found that complexes composed of Band 3 and 4.2 can be isolated from membranes with Triton X-100, and more recently Bennett and Stenbuck have discovered that Band 2.1 (ankyrin) seems to form complexes with a small fraction of Band 3 molecules [6].

It is clear that we are still in the preliminary stages of analyzing the number and types of protein-protein complexes that spectrin can form with other membrane proteins. So far it seems that spectrin may bind to or in the immediate vicinity of Bands 2.1, 3, 4.1, and possibly 4.2. The simplest conclusion to be drawn from these studies is that spectrin indeed has the capacity to bind to protein molecules attached to the inner surface of the ghost membrane. Whether this is the only mechanism or indeed even the most physiologically important way that spectrin attaches to the surface of the red cell membrane remains to be determined.

Spectrin Binds to Membrane Lipids

Although the evidence is strong that spectrin can bind to at least one specific protein on the inner surface of the membrane, there are also some intriguing experiments linking spectrin directly with certain membrane lipids. Some years ago spectrin was found to bind to the exposed surfaces of lipid molecules oriented in multilamellar liposomes [63], but these studies did not attract much attention since the possibility for artifactual adsorption was great. This approach has been reinvestigated more recently with interesting results. Spectrin binds to liposomal surfaces composed of acidic phospholipids and influences the thermotropic behavior of such liposomes by changing both the temperature and the enthalpy of the phase transitions [45]. Liposomes incubated with spectrin also show an unusual property when incubated with cal-

cium. Calcium causes liposomes of acidic phospholipids to aggregate into stacked lamellae and to fuse together, but the addition of spectrin to the liposomes "protects" them against this effect of calcium. Others have also shown that spectrin can somehow interfere with the binding of calcium and magnesium to liposomes composed of phosphatidyl serine [53]. No attempt was made to measure the affinity of spectrin for the liposomes in either study.

Direct evidence that spectrin may bind to phospholipids of the red cell membrane has been provided by chemical cross-linking studies of Marinetti and Crain [44]. These investigators have discovered that the normally nonpenetrating probe difluorodinitrobenzene (DFDNB) can pass across the membranes of intact red cells if the probe is administered in the form of a potassium-valinomycin complex. DFDNB added to red cells in this way cross-links phosphatidyl ethanolamine molecules to each other and to a protein complex which is rich in spectrin. Rough calculations indicate that between 19 and 25 phosphatidyl ethanolamine molecules become covalently-linked to each spectrin dimer. Almost equivalent amounts of phosphatidyl serine (13–17 molecules/molecule) were found linked to spectrin as well.

These results contradict to some extent the findings of Bennett and Branton who reported that spectrin did not bind to inside-out ghost vesicles if the vesicles were digested with proteases [4], since proteolytic digestion should not effect the binding of spectrin to lipids unless the lipids rearrange or become inaccessible to the added spectrin. It is conceivable that the amino phospholipids (PE and PS) which are normally present on the inner surface of the membrane flip across the bilayer, either during vesicle formation or as a result of spectrin depletion, as has been suggested by the experiments of Haest and co-workers [21].

Another possibility, which seems more attractive for the reasons described below, is that the binding of spectrin to lipids is of quite low affinity and would not be evident under the experimental conditions employed in the usual binding studies.

Does Spectrin Exist as a Spectrin-Actin Complex?

The preceding paragraphs review evidence which indicates that spectrin can bind to proteins on the inner surface of the red cell membrane and can also associate with specific acidic phospholipids. Either type of binding could explain the attachment of spectrin to the inner surface

of the red cell ghost membrane. However, a third alternative has also been proposed: Spectrin might exist as a submembranous network with a three-dimensional structure which is independent of its association with the overlying membrane. Some indication that such a structure might exist in red cells has been provided by studies using nonionic detergents to strip away membrane lipids and membrane proteins selectively. Steck has discovered that red cell ghosts can be suspended in Triton X-100 under conditions in which the ghost "shape" is not significantly modified even though the bulk of the membrane lipids and essentially all of the integral membrane proteins are lost into the medium. When the residues of these extracted ghost membranes are examined by scanning electron microscopy, a reticular pattern is observed [22]. This network obtained from Triton-extracted membranes is composed predominantly of spectrin and erythrocyte actin along with lesser amounts of other proteins. Sheetz and co-workers have further shown that the same approach can be used to isolate reticular structures from intact red cells [59]. The reticular networks (often referred to as Triton shells) isolated from red cells are more complicated in that they contain additional membrane proteins, including small amounts of Bands 3, 4.1, and 2.1.

Suggestions that spectrin and actin might form specific complexes in the red cell membrane have been proposed ever since it was realized that the two proteins are both extracted when red cell membranes are exposed to low ionic strength buffers. The large size of the spectrin polypeptide chains and their apparent association with actin were considered circumstantial evidence that the two proteins functioned as a myosin-actin complex. Since it is now obvious that spectrin is not at all myosin-like, other explanations for the interactions between them must be considered. Spectrin seems to have some effect on the *in vitro* polymerization of actin [64], and there is also indirect evidence that the degree of phosphorylation of spectrin may modulate this association [51]. On the negative side, many investigators have commented on their inability to find specific spectrin-actin complexes in red cell membranes which have been subjected to chemical cross-linking agents; only rarely have spectrin-actin complexes been produced and these have been in small amounts. These latter negative findings raise the possibility that red cell actin may be a vestigal protein left over from the erythroblast stage where it may have functioned in cell motility and enucleation. Geiduschek and Singer have recently shown that spectrin and actin are concentrated in different segments of the erythroblast plasma membrane during the

enucleation stage; actin lines that part of the membrane which surrounds the extruding nucleus, while spectrin remains with the membrane which eventually becomes the plasma membrane of the mature erythrocyte [19].

Purified muscle actin binds to red cell ghost membranes [11], and the binding is enhanced in the presence of crude spectrin preparations. Something in these preparations, possibly small amounts of F-actin, can serve as nucleation sites for the polymerization of G-actin onto the membrane surface. Nonpolymerized actin can also bind to spectrin which has been immobilized on Lytron beads [54]. At the moment there is no way of knowing whether these *in vitro* effects mimic the way that endogenous actin binds to the inner surface of the intact red cell membrane.

Although actin seems to be part of the "Triton shells" described above, enthusiasm for this model should not obscure the possibility that these shells could well be artifactual complexes created during the process of extracting integral proteins and lipids from the membranes. Since spectrin is present along the inner surface of the membrane in extremely high concentrations and is not solubilized by the detergent, the individual spectrin molecules could easily collapse into the cord-like structures seen with the scanning microscope, and if actin monomers or polymers are present one would expect these to be incorporated into the cords as well. Actin is not necessary for spectrin to bind to inside-out vesicles, as described above, and so far no one has yet succeeded in producing regular two-dimensional networks of spectrin and actin *in vitro*. The successful reconstitution of spectrin-actin lattices from purified components would go a long way to establishing the physiological significance of the spectrin-actin lattice model.

What Does Spectrin Do for the Red Cell Membrane?

Students of spectrin generally agree that the spectrin molecule, whatever its form, shape, or attachment sites, will probably prove to be involved in the following functions:

1. Regulate the topography of intramembranous proteins
2. Regulate erythrocyte shape and deformability
3. Stabilize the lipid bilayer.

Probably the first and most dramatic indication that spectrin may have an important role in regulating the distribution of transmembrane

proteins in the red cell was the experiment showing that the intramembranous particles undergo striking rearrangements if ghost membranes are incubated at pH 5.5 [52]. These intramembranous structures, which are believed to represent the intramembranous domains of transmembrane glycoproteins, are normally in a dispersed state in both intact red cells and in red cell ghosts, but a short incubation at pH 5.5 causes them to shift into tightly packed clumps. If the pH of the suspending medium is shifted back to above pH 7, the clumped particles resume their dispersed state. The mechanisms which underly this remarkable shift in these particles are not known, but there are good reasons for suspecting that spectrin plays some role. The pH of 5.5 which causes maximum particle aggregation is also near the isoelectric point of spectrin, and part of the spectrin network must be removed from the ghost membranes before particle aggregation can be induced [15]. On the basis of this latter observation Branton has suggested that the dispersed distribution of the intramembranous particles is maintained by the full complement of spectrin molecules, and the particles can shift laterally if the spectrin lattice is partially depleted and then subjected to a pH-induced conformational change. Similar changes in the distribution of intramembranous particles can be achieved by incubating red cell membranes with anti-spectrin antibodies [47]. Bivalent anti-spectrin antibodies bind to the inner surfaces of red cell ghosts and result in changes in the distribution of sialoglycopeptide binding sites on the exposed membrane surface. Monovalent antibodies which presumably bind with equal avidity at equivalent sites do not result in glycoprotein rearrangements.

Both sets of observations indicate that perturbation of the spectrin network influences the distribution of intramembranous structures. The simplest interpretation of these results is that the intramembranous structures are in some way constrained by an intact spectrin network, and when this network is modified corresponding changes occur in the distribution of the transmembrane proteins. However, these experiments do not tell us how shifts in spectrin actually influence the redistribution of the intramembranous particles, and several possibilities must be considered.

Spectrin could bind directly to cytoplasmic segments of the transmembrane glycoproteins, and if spectrin molecules move laterally in the plane of the membrane their attached proteins should also move with them. However, the available evidence indicates that spectrin does not bind directly to any of the major transmembrane glycoproteins. These conclusions, obtained from studies of spectrin binding to inside-out vesicles,

could conceivably be misleading if the inside-out vesicles do not retain the true topographical relationships of the transmembrane proteins, but if we accept the negative results at face value then we must also consider the possibility that spectrin influences the distribution of membrane proteins indirectly by modifying the distribution of the membrane lipids, which in turn influence the intramembranous segments of the proteins. If spectrin binds to specific membrane lipids, changes in the distribution of the spectrin could alter the distribution of the membranous particles. While this could be a credible explanation for the shifts in intramembranous particles observed after incubating red cell membranes in acid pH or after incubating them with bivalent anti-spectrin antibodies, it is more difficult to conceive how the particles could become redispersed after acidified membranes are incubated in alkaline media.

A third possibility is that the distribution of spectrin modifies the distribution of another set of membrane proteins, possibly the spectrin-acceptor proteins, i.e., Bands 2.1, 4.1, or other species, and these proteins in turn influence the distribution of the transmembrane glycoproteins. No experiments have yet been reported which bear on this question apart from cross-linking evidence which is certainly consistent with the presence of "link-able" contacts between these proteins.

Spectrin seems a logical candidate to influence the shape and deformability of the red cell membrane. It is one of the most abundant proteins of the membrane and its submembranous location puts it in the right place to stabilize the lipid bilayer and influence the shape and deformability. Spectrin itself could form a two-dimensional lattice stabilized by protein-protein interactions, and it is easy to see how this lattice would be a factor in influencing cell shape and be able to influence membrane deformability. There are many ways in which the simple biconcave shape of the red cell membrane can be modified, and most of these involve subtle shifts in the metabolic state of the red cell which seem to affect the aggregation state of the spectrin molecules [69, 60, 48]. The levels of ATP clearly influence the transition of the red cell membrane from the biconcave disc to the spiny echinocyte [69], and since ATP levels seem to influence spectrin aggregability [48] it is not too far-fetched to suggest that spectrin is involved in discocyte-echinocyte transitions. Red cells which have been incubated with calcium in the presence of the ionophore A23187 become noticeably rigid and their membranes retain stress-induced deformities long after control cells have "snapped-back" to the normal discoid shape [28]. Although the effects of increased levels of calcium inside cells are potentially quite complex, it would

be surprising if the rigidity of the membrane was not accompanied by changes in the spectrin network.

One of the more dramatic examples linking the state of the spectrin scaffolding to the shape of the red cell membrane has been provided by studies of sickled erythrocytes. The spectrin-actin complex generated by Triton extraction of sickled erythrocytes is also sickle shaped, and it remains so in spite of the fact that the extracted membrane is largely free of hemoglobin [38]. Thus once the sickled shape is assumed by red cells containing hemoglobin S, and presumably hemoglobin S is the initiating agent, it appears that the abnormal shape of the membrane is retained by the spectrin-actin network. The rigid, sickled shape of the spectrin residue could be related to an increase in calcium in these cells or to oxidative cross-linking reactions.

It is likely that other red cell defects involving changes in shape but without detectable defects in hemoglobin may well prove to be due to primary defects in spectrin itself. Many investigators assume that the spectrin network in hereditary spherocytosis will also prove to be defective [26]. Preliminary studies of red cells from mice with a form of hereditary spherocytosis indicate that the affected cells contain only a small fraction of the spectrin found in normal cells [20].

The spiny processes of the echinocytic red cells seem to have a normal amount of submembranous spectrin [73], but endocytosis vesicles of both neonatal red cells [65] and adult red cells [23] seem to lack a spectrin network. These findings are consistent with the idea that the spectrin serves to stabilize large expanses of lipid bilayer and other mechanisms facilitate the phospholipid shifts required to form small vesicles and permit fusion between opposed membranes. It is interesting that anti-spectrin antibodies inhibit the fusion of red cell ghost membranes caused by Sendai virus [58, 32].

In line with the idea that the spectrin network serves to stabilize lipid bilayers is the recent suggestion that spectrin may even be involved in maintaining the asymmetrical localization of different phospholipids. Phospholipid asymmetry in human red cells is modified if spectrin is exposed to oxidizing agents [21]. Although these experiments are complicated and may well involve a number of mechanisms, it is intriguing to consider the possibility that spectrin binds the bulk of the PS and PE molecules and thereby confines them to the inner surface of the red cell bilayer. When spectrin is perturbed either chemically, as in the case of red cells treated with oxidizing agents, or by other physical means the interactions between the overlying phospholipids could be

disturbed to the extent that they either move away or flip across the bilayer.

How Does Spectrin Work?

It seems safe to assume that spectrin plays a major role in regulating both the stability and deformability of the red cell membrane. This membrane must be able to withstand the shearing pressures generated in the heart during systole and, shortly thereafter, be able to permit the red cells to insinuate themselves through narrow capillary loops in the different tissue spaces, and do this over a life span which exceeds that of any other blood cell. Thus this membrane must be both rigid and pliable depending upon the circumstances.

Red cell membranes are probably not stabilized by an unlimited end-to-end polymerization of spectrin units as suggested earlier. It is also unlikely that sliding filaments of spectrin and actin control red blood cell shape changes, or that both membrane stability and deformability can be satisfied by a simple, fixed, two-dimensional lattice of spectrin and actin.

If spectrin tetramers do indeed exist as flexible rods *in vivo* they might be able to provide both stability and deformability on the basis of differential binding to both proteins and lipids. If spectrin rods bind simultaneously to both high affinity protein carriers and to low affinity lipid sites, they could function as "floating-ribs" by maintaining tight associations with membrane proteins and loose associations with specific lipids and with neighboring spectrin tetramers. The binding of spectrin to specific lipids, probably PE and PS, could cause the lipids to cluster into loosely packed domains, and such bound lipids might be expected to remain fixed to the inner surface of the bilayer. It is perhaps more than fortuitous that the binding of spectrin to the amino phospholipids could serve a number of purposes. The binding of spectrin to PS and to PE might provide stability to the lipid bilayer and at the same time prevent these lipids from coming in contact with extracellular calcium and the serum proteins involved in blood clotting. The millimolar amounts of calcium in the extracellular fluids could cause phase separation of the lipids and/or promote membrane fusion. Neither effect would serve a useful purpose for the normal blood cell.

The notion that spectrin tetramers might exist as "floating ribs" immediately beneath the lipid bilayer depends upon the presence of sev-

eral different binding domains on the spectrin molecule. Spectrin tetramers would have to be anchored to the membrane via tight associations with a small number of specific carrier proteins, and they would have to have multiple sites to which specific phospholipids might bind with low affinity. The beta subunit of spectrin has at least one site which binds to a protein called ankyrin, and this may be the principal high affinity "anchoring" site. The evidence that spectrin has multiple binding sites for specific phospholipids is less convincing, but there are several promising leads which suggest that this may well be the case. In order for complexes composed of spectrin, acceptor proteins, and lipids to provide long range stability to the red cell membrane spectrin molecules would also have to have the capacity to form loose associations with each other. Since spectrin in solution invariably forms aggregates if the protein concentration is greater than 5 mg/ml (10 μM), it is likely that membrane-bound spectrin also forms similar complexes, since the effective concentration of spectrin *in situ* is approximately tenfold higher ($\sim$100 μM). Oligomers of spectrin are also produced routinely by incubating intact red cells or ghosts with chemical cross-linking reagents.

I thank Jon Morrow and Dave Speicher for their critical reviews of this manuscript.

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