Anatomy of the red cell membrane skeleton: unanswered questions

Samuel E. Lux, IV

1Dana-Farber/Boston Children’s Cancer and Blood Disorders Center and Department of Medicine, Boston Children's Hospital, Boston, MA 02115
Abstract

The red cell membrane skeleton is a pseudohexagonal meshwork of spectrin, actin, protein 4.1R, ankyrin, and actin-associated proteins that laminates the inner membrane surface and attaches to the overlying lipid bilayer via band 3-containing multi-protein complexes at the ankyrin and actin binding ends of spectrin. The membrane skeleton strengthens the lipid bilayer and endows the membrane with the durability and flexibility to survive in the circulation. In the 36 years since the first primitive model of the red cell skeleton was proposed, many additional proteins have been discovered and their structures and interactions have been defined. But myriad questions about the skeleton’s structure remain and almost nothing is known of its physiology. Questions such as the structure of spectrin in situ, the way spectrin and other proteins bind to actin, how the membrane is assembled, the dynamics of the skeleton when the membrane is deformed or perturbed by parasites, the role lipids play, variations in membrane structure in unique regions like lipid rafts, and so on. This knowledge is important because the red cell membrane skeleton is the model for spectrin-based membrane skeletons in all cells, and because defects in the red cell membrane skeleton underlie multiple hemolytic anemias.
History

Modern knowledge of the red cell plasma membrane and its membrane skeleton began with Marchesi and Steer’s identification of spectrin in 1968. Prior to that year, almost the only thing known about membranes was that they contained a lipid bilayer. Indeed, there was a period in the 1960s when it was believed that red cell membranes contained only a single 22.5 kD protein called “structural protein”. The fertile decade following the discovery of spectrin and the more or less coincident introduction of SDS polyacrylamide gel electrophoresis as an analytic tool, led to the isolation and characterization of several major red cell membrane proteins. The first simple model of the membrane skeleton appeared 36 years ago (Figure 1). While it contained the core elements of the modern model, a great deal has been learned in the intervening decades (Figure 2). Hundreds of individuals have contributed to our knowledge of the red cell membrane skeleton but a few deserve special recognition, including: Peter Agre, David Anstee, G. Vann Bennett, Daniel Branton, Lesley Bruce, Jean Delaunay, David Discher, Velia Fowler, Patrick Gallagher, Walter Gratzer, Philip Low, Vincent Marchesi, Narla Mohandas, Jon Morrow, Jeri Palek, Luanne Peters, David Speicher, Ted Steck, and Michael Tanner.

This review will focus on our current understanding of the anatomy of the membrane skeleton, particularly its spectrin-actin core, and on some of the key unanswered questions. Because of space limitations, many important aspects of the red cell membrane are not discussed. These include: integral membrane proteins that are not attached to the skeleton, the lipid bilayer, membrane biogenesis, post-translational modifications of the membrane, red cell aging, and mouse and human membrane diseases. For interested readers, a few other recent reviews are recommended.

The Red Cell Membrane Skeleton

The red cell membrane contains about 20 major proteins and at least 850 minor ones. Some of the important ones are described in Table 1. The integral membrane proteins are organized into macromolecular complexes centered on band 3, the anion exchange channel. Most of the peripheral
membrane proteins form the membrane skeleton, a 40 nm to 90 nm thick protein meshwork that laminates the inner membrane surface. The skeleton is composed principally of spectrin, actin and its associated proteins (tropomyosin, tropomodulin, adducin and dematin), protein 4.1R and ankyrin.

**Spectrin**

Erythrocyte spectrin is a long, flexible, worm-like protein composed of two parallel chains (α- and β-spectrin) oriented in opposite directions. Each chain contains multiple spectrin-type repeats with specialized functional domains at the “head” end for spectrin dimer-tetramer association and for ankyrin-1 binding, and domains at the “tail” end for binding to protein 4.1R, protein 4.2, short filaments of actin and other proteins. The details of spectrin structure are shown in Figure 3. On average, six spectrins bind per actin filament, leading to a pseudohexagonal arrangement.26

Isolated α- and β-spectrin chains bind to each other electrostatically at a pair of nucleation sites formed by repeats near the spectrin tail (Figure 3A). Once connected, the rest of the α- and β-chains zip together, coiling around each other roughly every four27 repeats. The side-to-side interactions beyond the nucleation site are relatively weak,28 allowing the two chains to slide past each other when the spectrin molecule flexes and extends during membrane deformation.

Spectrin α- and β-chains associate with each other at the “head” end. Basically, the complementary partial repeats (α0 and β17) at the end of each chain join to form a complete three-helix repeat: α0/β17 (Figure 3A). In spectrin αβ-dimers, the longer α-chain folds back on itself, so the α0/β17 repeat interacts with repeat α4 and the α1 and α3 repeats contact each other (Figure 3A).13 In the α2β2-heterotetramers there are three such side-to-side interactions between the longer α-chains (Figure 3C). These lateral interactions, though weak, contribute to self-association. Higher oligomers (hexamers, octamers, etc) form by the same mechanism and can also be seen on the membrane.26,29,30 Even though nearly 95% of the spectrin is in the tetramer or oligomer forms,31 spectrin self-association is uniquely weak in the red cell.32 Tetramers dissociate and reform under physiologic conditions and this is greatly accentuated when the
membrane is distorted by shear forces.\textsuperscript{33} This may be an evolutionary accommodation to permit the enormous distortions the red cell undergoes in parts of the microvasculature.

Spectrin is a highly flexible molecule but it is not certain how it achieves its flexibility since crystal structures of tandem repeats shown a continuous helix across the junction between repeats (Figure 3B). There is evidence that the helical linker may bend in certain directions at the junction\textsuperscript{34} and that helices within some repeats may ‘melt’ and rearrange, shortening the repeat.\textsuperscript{34} In addition, some of the repeats are unstable at physiological temperatures (starred repeats in Figure 3A),\textsuperscript{23} and may partially unfold when red cells deform, which would contribute to flexibility.\textsuperscript{35}

The Actin Protofilament and Its Associated Proteins

\textit{Actin}

Red cells contain short, double helical, filaments of nonmuscle or \(\beta\)-actin, termed “protofilaments” (Figure 2). These lie roughly parallel to the membrane plane (±20 degrees) and are randomly oriented.\textsuperscript{36} There are about 30,000-40,000 protofilaments per red cell, with 6-8 actin monomers in each of the two strands.

Not much is known about how these unique actin filaments form but some hints are emerging from defects in actin assembly. Mice that lack both Rac1 and Rac2 GTPases,\textsuperscript{37} or lack Hem-1, a member of the WAVE complex that regulates F-actin polymerization,\textsuperscript{38} develop hemolytic anemias with misshaped red cells, and clumped or irregular skeletons. This suggests that Rac GTPases and Hem-1 help organize or regulate the red cell membrane skeleton, acting through pathways that stimulate actin polymerization in many cell types. It is unclear whether such regulation occurs dynamically in the mature red cell or just during erythropoiesis.

\textit{Tropomyosin}

Erythrocyte tropomyosin (TM) is a long rod-like dimer of \(\alpha\)- and \(\gamma\)-TM isoforms (hTM5b and hTM5, respectively).\textsuperscript{10} One TM dimer binds to each of the two strands in the actin protofilament (Figure 2).
Binding is Mg\(^{2+}\)-dependent. The red cell TM isoforms also bind to tropomodulin (see below). Red cell membranes that lack TM are markedly more fragile than normal\(^{39}\) and only endogenous TM, not the longer muscle isoforms, can restore stability. Red cell TM is just long enough (~34 nm) to cover the estimated 7 actin monomers in one strand of the F-actin protofilament, which supports the idea that tropomyosin serves as a molecular ruler, along with actin capping proteins, in assembling the filament\(^{40}\) and strengthens the filament after assembly.

In yeast, formins—a family of proteins that nucleate actin filaments—direct which TM isoform associates with an actin filament.\(^{41}\) It will be interesting to see if that is also true in erythroid precursors, and if formins play a role in protofilament formation.

**Tropomodulin-1**

Tropomodulin-1 (TMod1) has two functions: it caps the pointed or slow-growing end of actin filaments (Figure 2) and binds tropomyosin, which greatly strengthens actin capping.\(^{42}\) There are 30,000 copies per red cell or one per actin protofilament. One TMod1 binds to both actin strands and both TMs in the actin protofilament.\(^{42}\) Mice that lack TMod1 in their red cells have variable actin protofilament lengths and irregular holes in the membrane skeleton.\(^{43}\) These mice also have increased TMod3 in their red cells, which may partially compensate. TMod3 is a protein that plays multiple roles in definitive erythropoiesis, erythroblast binding to so-called “nurse” macrophages, and enucleation\(^{44}\) but is not normally present in mature red cells.

**Protein 4.1R**

Protein 4.1R promotes spectrin-actin binding and helps attach the membrane skeleton to the membrane. The first of these functions is especially important. Under physiologic conditions, erythrocyte spectrin binds very weakly to F-actin. Protein 4.1R binds to both spectrin and actin and generates a high affinity ternary complex.\(^{45}\) The cofactor activity is contained in a 10-kd spectrin-actin binding domain (SABD) in the middle of the molecule\(^{46}\) (Figure 4). The actin-binding site in the SABD is straddled by a two-part
spectrin-binding site in a single linear peptide (Figure 4C). Red cells that lack protein 4.1R are very fragile, but their membrane strength is normalized by the SABD peptide. Unfortunately, the three-dimensional structure of the SABD within protein 4.1R is unknown. Solving the complete structure of this important protein is a major need in the field.

The other important 4.1R domain is the N-terminal, globular membrane binding domain (MBD). It binds glycophorin C, protein p55, band 3 and calmodulin in separate, well-defined areas (Figure 4B). These interactions are discussed briefly in the section on the Actin Junctional Complex.

Adducin

Adducin is a complex, multifunctional protein containing an α-subunit, and either a β-subunit or less often a related γ-subunit. The adducin subunits contain a globular head domain and an extended, flexible tail. The predominant form is probably the αβ heterodimer. There are 30,000 copies of the dimer per red cell.

As suggested by the number of copies, adducin caps the fast growing or “barbed” end of the actin protofilaments in red cells (Figure 2). It also recruits spectrin to neighboring sites on actin, which enhances the capping about 10-fold. These interrelated functions require a domain at the end of the adducin tail. Both functions are regulated by calmodulin and by several protein kinases. The effects of phosphorylation are complex and it isn’t clear if they are physiological important. Indeed, the importance of adducin itself is uncertain, since mice that lack the protein have only mild hemolysis and spherocytosis.

Adducin also helps attach the membrane skeleton to the lipid bilayer through interactions with the anion exchange channel (band 3) and glucose transporter-1 (Figure 2). The band 3 binding site is located in the tails of α- and β-adducin. Whether adducin binds dimers or tetramers of band 3 and whether it binds one or two of each is unknown.
**Dematin**

Dematin is a monomeric, actin binding protein with two actin binding sites: one in its headpiece and one in its tail.\(^5^8\) Dematin was first identified by it’s ability to bundle actin filaments into cables,\(^5^9\) but in mature red cells, where there are no such cables, dematin has two functions. First, unmodified dematin binds to spectrin and enhances spectrin-actin binding.\(^6^0\) This function is lost when dematin is phosphorylated by protein kinase A.\(^5^8,6^0\) Second, dematin binds to the glucose transporter\(^5^7\) and helps attach actin to the membrane.

**How Does Spectrin Bind to the Actin Protofilament?**

Surprisingly little is known about this central question. Actin and protein 4.1 bind to two tandem calponin homology (CH) domains at the N-terminal- or tail-end of \(\beta\)-spectrin (Figure 3A). The CH1 domain is constitutively active but the CH2 domain is incipient.\(^1^8\) It is unmasked by removal of a blocking helix or by phosphatidylinositol-4,5-bisphosphate (PIP2), a lipid involved in many signaling pathways.\(^1^8\) How, or even if, PIP2 regulates the CH2 domain in the native red cell is not known.

The neighboring calmodulin-like EF hand domain of \(\alpha\)-spectrin is also important. Deletion of the last 13 amino acids of the domain in a mini-spectrin or in the severely hemolytic sph\(^1^J\) mouse nearly ablates actin binding.\(^2^1\)

The relationships of the EF hand domain, the calponin homology domain and F-actin are not known in spectrin, but probably resemble those in better studied spectrin family members such as \(\alpha\)-actinin,\(^2^4,2^5,6^1\) utrophin,\(^6^2\) dystrophin, fimbrin, and plectin (Figure 3C). The problem is, these proteins bind actin in different ways. Sometimes the CH domains are in contact with each other (closed position), and sometimes they are extended and separated from each other (open position). In some analyses only CH1 binds to actin;\(^2^5\) in others both CH1 and CH2 bind.\(^2^4,6^1\) The EF hand domain is always in contact with the CH domains and not in contact with actin, so it presumably regulates the CH domains. The fact that the \(\alpha\)-actinin CH domains adopt both open and closed positions\(^2^4\) and that utrophin also has two modes of
binding\textsuperscript{62} suggests that the CH domains in spectrin might also do so and that this might be regulatory (convert weak binding to strong binding, for instance). Perhaps protein 4.1R and/or the EF domains act by converting the CH domains to a high affinity conformation. Spectrin could occupy up to two actins in the actin filament given the stoichiometry (6 spectrins/$\sim$14 actin monomers – see Table 1), so a variety of arrangements are possible. In any event, these are clearly key questions that need to be answered going forward.

Another obvious problem that is rarely mentioned is that spectrin is in a planar array, but the spectrin binding sites on the helical actin protofilament must be in a helical array. Spectrin doesn't hang down into the cytoplasm or stick up into the lipid bilayer, so if all the spectrin binding sites on actin are saturated, either the actin-binding end of spectrin must be flexible enough to curve around the actin filament or spectrin must be able to bind to actin in more than one way.

**What is the Structure of Spectrin on the Membrane?**

Electron micrographs of purified spectrin tetramers\textsuperscript{63} or spectrin filaments in fully stretched skeletons\textsuperscript{26} (Figure 5A) show a spaghetti-like molecule with an end-to-end length of up to 200 nm. This is how spectrin is usually depicted in membrane models. However, the filaments of native, unstretched skeletons are much more compact (Figure 5B) and simple calculations show that the average distance between actin filaments—i.e., the length of a spectrin tetramer—is only 60-70 nm in vivo.\textsuperscript{8,66} This corresponds roughly to the length of presumed spectrin filaments in native skeletons.\textsuperscript{29,30,65} It isn’t known how spectrin folds in this resting state. The best evidence comes from visually enhanced electron micrographs of spectrin in partially expanded skeletons (Figure 5C and D). These suggest that $\alpha$- and $\beta$-spectrin are coiled about each other in a two-start helix with approximately 10 turns per spectrin tetramer\textsuperscript{27} (Figure 5E). Some micrographs show relatively straight spectrin filaments;\textsuperscript{27} others show some kinking.\textsuperscript{30} This may reflect whether the filaments are under tension. In this model, spectrin molecules extend and contract along the helical axis by varying the pitch and diameter of the double strand (Figure 5E).\textsuperscript{27} This spring-like behavior is presumably critical to the elastic behavior of the membrane.
However, there is evidence for other models—such as having spectrin molecules adopt a random worm-like configuration, or having helical segments of the repeats that ‘melt’ and extend under tension or that simply unfold when stressed. The fact that previously occluded cysteines can be labelled when red cells are deformed by shear is evidence that some repeats unfold and that spectrin may even detach from ankyrin or actin when stretched. Since the structure of spectrin affects its mechanical properties as well as the spatial relationships of all the other proteins that bind to it, defining its structure in vivo, at rest and when deformed, is a high priority.

**Attachment of the Membrane Skeleton**

**Ankyrin**

Erythrocyte ankyrin (ankyrin-1 or ankyrin-R) links spectrin to a complex of band 3 and other proteins in the lipid bilayer. The protein has three domains (Figure 6). The membrane domain is composed entirely of ankyrin repeats. It is a remarkably open, spiral structure, somewhat like a twisted sickle, that encircles and binds band 3 tetramers (Figure 6B). Whether band 3 spontaneously forms tetramers and then attracts ankyrin, stabilizing the tetramers, or whether ankyrin, which has two binding sites for band 3 (Figure 6A), independently binds two band 3 dimers (i.e., a “dimer of dimers”), is unresolved. The fact that a stable subpopulation of band 3 tetramers can be isolated from nonionic detergent extracts of ghosts argues for the former. But, the structure of a band 3 tetramer binding to the full membrane domain has not been solved and at least one recent paper argues that band 3 dimers must bind independently, so the question remains.

The ankyrin-spectrin binding site is well defined. The ZU5A subdomain of ankyrin binds to a special site created by the junction of the 14th and 15th repeats within β-spectrin (Figure 6C). Only one ankyrin binds per tetramer, perhaps for steric reasons. Ankyrin binding promotes spectrin tetramer and oligomer formation by about 10-fold and vice-versa. Ankyrin attachment to band 3 also strengthens spectrin self-
Presumably, both of these mechanisms help regulate the relatively tenuous self-association interaction.

**Band 3**

Erythrocyte band 3, or officially SLC4A1, is the major red cell membrane protein, with about 1.2 million copies per cell. Functionally, it is two proteins: an N-terminal, cytoplasmic, peripheral membrane protein that is a key attachment site for the membrane skeleton, glycolytic enzymes and deoxyhemoglobin; and a C-terminal integral membrane protein that forms the red cell anion exchange channel and aids CO$_2$ transport (Figure 7).

The glycolytic enzymes form a metabolic complex (“metabolon”) extending from phosphofructokinase (PFK) through lactic dehydrogenase (LDH). Three of the enzymes bind to the N-terminus of band 3: PFK, aldolase and glyceraldehyde-3-phosphate dehydrogenase (G3PD). The others bind indirectly as part of the complex. The enzymes are inactive when bound but they are displaced and activated by deoxyhemoglobin, which also binds to the N-terminus, or by phosphorylation of two tyrosines within the binding sites (Figure 7). The enzymes and their ATP product are located in discrete clumps along the membrane, which supports the idea of a physical metabolon. Conditions that displace the enzymes from the membrane activate glycolytic fluxes by 45% in intact red cells, which supports a functional one.

As noted earlier, ankyrin binds to band 3 tetramers. The acidic N-terminus and two loops on the surface of band 3 are involved in the binding (Figure 7). As with the glycolytic metabolon, ankyrin is displaced from band 3 by deoxyhemoglobin. The affinity of deoxyhemoglobin is weak, but the concentration of hemoglobin is so high in red cells that roughly half of the band 3 molecules are bound to deoxyhemoglobin when red cells are deoxygenated. Loosened ankyrin constraints could improve blood flow in hypoxic areas, but with the risk that red cells subject to prolonged deoxygenation, such as those trapped in the spleen, might suffer membrane vesiculation. (One wonders if this is the long sought mechanism for splenic conditioning in hereditary spherocytosis?)
The Ankyrin Complex

Ankyrin and band 3 are part of the multiprotein ankyrin complex (Figure 2). From the amounts of each protein in the red cell (Table 1), the complex is estimated to contain: one ankyrin, one band 3 tetramer, two glycoporphin A or B dimers or heterodimers, two protein 4.2 molecules, and one Rh complex (a trimer of RhAG combined with Rh D and RhCE). CD47, LW and the proteins in the glycolytic metabolon are also components but they are present in less than stoichiometric amounts (Table 1) and so must only contribute to a subset of complexes. One wonders how many such subsets there are? Do they have constant composition or are they in a dynamic equilibrium? And are the rare complexes that contain CD47 and LW localized to specific membrane subdomains?

As detailed in Table 1 and Figure 2, there are multiple interactions among proteins in the ankyrin complex. Some of these are critical. Red cells lacking band 3 also lack protein 4.2 and glycophorin A, for example.\textsuperscript{89} And, red cells lacking protein 4.2 nearly lack CD47.\textsuperscript{90} A consequence of this promiscuity is that missense mutations that interfere with a single interaction within the ankyrin complex have little effect. Hereditary spherocytosis is caused by defects in spectrin, ankyrin, band 3 or protein 4.2 that impair ankyrin complex formation, and almost all the known mutations diminish the concentration of these proteins rather than block their binding functions.

The Actin Junctional Complex

The actin protofilament and its associated proteins are also attached to the membrane. For many years this was believed to occur exclusively via a ternary complex of protein 4.1R, p55 and glycophorin C or D. Each of these proteins interacts with the other two and the interaction sites are well mapped.\textsuperscript{47,91,92} However, it was never clear why, if this was an important skeleton attachment and the sole attachment at the actin binding end of spectrin, patients with complete absence of glycophorin C and D had, at most, very mild hereditary elliptocytosis.

During the past few years it has become clear that the actin junctional complex is more complex and, like the ankyrin complex, is centered on band 3 (Figure 2). First, protein 4.1R and ankyrin compete for
binding to band 3, which suggests they bind to separate band 3 populations. The interaction with 4.1R is known to be important since zebrafish lacking band 3 can be rescued by expression of mouse band 3 but only if the mouse band 3 contains the 4.1R binding sites. Second, protein 4.2, which requires band 3 for incorporation into the membrane, binds to the EF hand domain at the actin-binding end of spectrin. Third, adducin binds to band 3, which definitely localizes a subpopulation of band 3 to the neighborhood of actin (Figure 2). The latter interaction is important since the membrane fragments if the connection is severed.

The stoichiometry of the actin junctional complex is uncertain. On average, six spectrins and six proteins 4.1R bind per actin (Figure 8), and there is enough band 3, glycophorin A, glycophorin C/D (GPC/D), and probably enough Glut1 and stomatin to supply six complexes per actin filament (Table 1). But, there is only one copy of adducin per actin filament and only three copies of dematin if it is a monomer as recent studies indicate. Similarly, while protein 4.1R, GPC/D and protein p55 are usually pictured as a three part complex, there is only enough p55 for one copy per actin filament if it is a dimer, as some data suggest. And there is only about one copy of protein 4.2 available if 240,000 copies (one per band 3 dimer) are tied up in the ankyrin complex. The actin junctional complex also contains enzymes in the glycolytic metabolon and the blood group proteins Kx/Kell and DARC/Duffy, though the amounts of these proteins are insufficient for even one copy per complex.

So, it is uncertain at the moment whether the actin junctional complex contains only a single band 3 dimer, interacting with just one or two of the six spectrin-4.1R dyads, or whether it is a larger complex containing three to six band 3 dimers that interact with all the spectrins and proteins 4.1R. Both possibilities are depicted in Figure 8. Recent evidence favors a larger complex, but the data are not conclusive. If so, it is not clear whether all or just a subset of band 3 molecules bind to adducin.

In either case, as shown in Figure 8, one of the interesting and under appreciated facts is that the ankyrin and actin junctional complexes are quite close to each other in situ on the membrane and must often collide, especially during red cell deformation, but perhaps even when the cell is at rest. This raises
the interesting possibility that proteins like band 3, protein 4.1R, protein 4.2, and adducin, which have binding partners in both complexes, may sometimes switch allegiances. Since some proteins compete for the same binding sites—for example, as noted, protein 4.1R displaces ankyrin from band 3—this could be a regulatory process.

**Distribution of Band 3**

Of the ~1.2 million band 3 monomers per red cell, 40% are tetramers bound to ankyrin. Depending on whether the actin junctional complex contains an average of one or six band 3 dimers, an additional 7% to 40% of the band 3 dimers are located there. Recent experiments comparing normal mouse erythrocytes to α-adducin-deficient erythrocytes suggest that roughly 33% of the band 3 molecules are immobilized by adducin, which favors a larger complex. The remaining band 3 dimers (25-30%) are believed to be diffusing freely in the lipid bilayer, constrained for the most part by the boundaries of the spectrin corrals, which average less than 100 nm in size. This is remarkably similar to the values of 25% to 29% freely diffusing band 3 measured in normal red cells using different methods.

It is important to remember that some of the minor proteins found in band 3 complexes can only exist in a subset of the complexes—probably multiple different subsets—and that the lipid bilayer is heterogeneous, with areas like the lipid rafts where certain proteins concentrate. So, the band 3 complexes must be more heterogeneous than these three states imply. Analyses of band 3 mobilities support this conclusion.

**The Future**

A great deal has been learned about the structure of the red cell membrane and the membrane skeleton in the 36 years since the first model was published (Figure 1). I sometimes hear people say that the red cell membrane is no longer an active area of hematology research, but I disagree. We have the broad outlines of how the membrane is organized (Figure 2), but we know few of the details, and what we know is about a static structure. Many questions remain (Table 2). We don’t know how the skeleton is constructed...
during erythropoiesis. We know very little about the dynamics of the skeleton—how labile individual bonds are, how the skeleton responds to capillary deformation, how the skeleton is disassembled and reassembled during invasion by malaria or other parasites, and so forth. And as discussed above, we don’t know how spectrin is folded in the intact skeleton or how the proteins that form the actin junctional complex are organized. Or how spectrin binds to actin. We don’t know if band 3-binding proteins like 4.1R, 4.2 and adducin switch from one band 3 complex to another given the apparent proximity of the complexes at each end of spectrin (Figure 8). We don’t understand why the same repeats in different spectrins are so similar if they just serve as spacers. Do they have binding or mechanical functions that we don’t yet know about? We don’t know what roles post-translational changes like phosphorylation, fatty acylation, methylation, glycation, hydroxylation and ubiquitination, or Ca$^{2+}$-binding and ATP-binding play. And, we don’t understand the interactions between the membrane skeleton and the overlying lipids, which will likely have regulatory as well as structural functions. This is only a partial list—some other questions are itemized in Table 2—but I hope my point is clear. Since the red cell membrane skeleton is the paradigm for studies of spectrin-based membrane skeletons in all cells, and since we now know that spectrin and other membrane skeletal proteins invest internal organelles and some transport vesicles as well as plasma membranes, and even play a role in nuclear organization, it is more important than ever to understand the structure and function of the red cell membrane skeleton.
Acknowledgments

The author regrets that important papers from many talented individuals who have made significant contributions to red cell research could not be discussed or cited due to space limitations. I gratefully acknowledge the camaraderie and friendship of a large number of colleagues in the red cell membrane field, who over many years have enriched my career and aided my research.

S. E. Lux wrote the review and designed the figures and tables.

The author has no conflict of interest to declare.
References


33. An X, Lecomte MC, Chasis JA, Mohandas N, Gratzer W. Shear-response of the spectrin dimer-


54. Manno S, Takakuwa Y, Mohandas N. Modulation of erythrocyte membrane mechanical function...


64. Byers T, Branton D. Visualization of the protein associations in the erythrocyte membrane...


84. Sterling D, Alvarez BV, Casey JR. The extracellular component of a transport metabolon. Extracellular loop 4 of the human AE1 Cl⁻/HCO₃⁻ exchanger binds carbonic anhydrase IV. *J Biol


| Protein       | Molec-
<table>
<thead>
<tr>
<th></th>
<th>-ular Mass (kd)*</th>
<th>Monomers/ Cell†</th>
<th>Oligomeric State</th>
<th>Oligomers in Ankyrin Complex</th>
<th>Oligomers in Actin Jct Complex</th>
<th>Functions in Mature Erythrocyte Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>41.6</td>
<td>500</td>
<td>Oligomer (~14)</td>
<td></td>
<td></td>
<td>1 Forms ~37 nm long filaments that serve as a molecular junction in the membrane skeleton. Binds spectrin with the aid of protein 4.1R. Binds tropomyosin, tropomodulin, adducin, dematin and chorein</td>
</tr>
<tr>
<td>α-Adducin</td>
<td>81.0</td>
<td>30</td>
<td>Heterodimer/ tetramer</td>
<td></td>
<td></td>
<td>1 Caps the fast-growing (barbed) end of actin and recruits spectrin to nearby sites on actin. Links actin junction to band 3, Glut1 and maybe stomatin. Binds chorein.</td>
</tr>
<tr>
<td>β-Adducin</td>
<td>80.7</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldolase A</td>
<td>39.4</td>
<td>20</td>
<td>Tetramer</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1 Forms glycolytic complex on band 3 with PFK, enolase, G3PD, PK and LDH</td>
</tr>
<tr>
<td>Ankyrin-1§</td>
<td>206.0</td>
<td>124 ± 11</td>
<td>Monomer</td>
<td>1</td>
<td>3</td>
<td>Attaches β-spectrin and the membrane skeleton to band 3 and to protein 4.2 and RhAG in the ankyrin-linked band 3 complex</td>
</tr>
<tr>
<td>Band 3 (SLC4A1)</td>
<td>101.8</td>
<td>1200</td>
<td>Dimer or tetramer</td>
<td>2 (dimer)</td>
<td>6 (dimer)</td>
<td>1-6 Anion exchange channel (especially HCO₃⁻ and Cl⁻) with the aid of CAII and CAIV. Forms membrane complexes with GPA, GPB, the Rh complex, Glut1, stomatin and other proteins that are linked to spectrin via ankyrin-1 and protein 4.2, and to the actin junctional complex via protein 4.1R, protein 4.2, p55, adducin, and Glut1. Binds peroxiredoxin. Forms a glycolytic metabolon with G3PD, PFK, PGK, aldolase, LDH and PK. Binds deoxyhemoglobin. Carries Ii and many other blood group antigens.</td>
</tr>
</tbody>
</table>

For personal use only.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Mw</th>
<th>mr</th>
<th>State</th>
<th>Multiplicity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic anhydrase II</td>
<td>29.1</td>
<td>100</td>
<td>Unknown</td>
<td></td>
<td>Attaches to the outside (CAIV) and inside (CAII) surfaces of band 3, the HCO₃⁻ channel. Catalyzes interconversion of CO₂ and HCO₃⁻, which aids in HCO₃⁻ transport and CO₂ elimination.</td>
</tr>
<tr>
<td>Carbonic anhydrase IV</td>
<td>33.1</td>
<td></td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>37.1</td>
<td>6-10</td>
<td>Unknown</td>
<td>&lt;1</td>
<td>Binds to protein 4.1R and maybe to ankyrin. Multifunctional protein in nonerythroid cells. Function in the red cell is unknown.</td>
</tr>
<tr>
<td>CD47</td>
<td>33.3</td>
<td>17</td>
<td>Monomer</td>
<td>&lt;1 &lt;1</td>
<td>Binds to protein 4.2 and to the Rh-complex (RhAG/RhD/RhCE) within the ankyrin-linked band 3 complex. Inhibits phagocytosis.</td>
</tr>
<tr>
<td>DARC/Duffy</td>
<td>35.6</td>
<td>6-13</td>
<td>Unknown</td>
<td>&lt;1</td>
<td>Chemokine receptor. Carries the Duffy (Fy) blood group antigen. Receptor for <em>P. vivax</em>. Function in normal red cell unknown.</td>
</tr>
<tr>
<td>Dematin</td>
<td>43.1 &amp; 45.5</td>
<td>130</td>
<td>Monomer</td>
<td>3</td>
<td>Binds and bundles actin filaments. Facilitates spectrin-actin binding. Helps attach actin to the membrane via Glut1</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>35.9</td>
<td>500</td>
<td>Tetramer</td>
<td>1 3</td>
<td>Forms glycolytic metabolon on band 3 with aldolase, PFK, enolase, PK and LDH.</td>
</tr>
<tr>
<td>Glucose transporter-1</td>
<td>54.1</td>
<td>200-700</td>
<td>Dimer &amp; tetramer</td>
<td>? ?</td>
<td>1-6 (dimer) Transports glucose. Transports L-dehydroascorbic acid when bound to stomatin. Links actin to the membrane via interactions with dematin, adducin and band 3.</td>
</tr>
<tr>
<td>Glycophorin A</td>
<td>14.3</td>
<td>1000</td>
<td>Homo or heterodimer</td>
<td>2 6</td>
<td>1-6 Binds band 3 in the ankyrin-linked band 3 complex, and maybe to RhAG. May facilitate band 3 transfer to the membrane. Carries MNSsU blood group antigens and much of red cell's negative surface charge.</td>
</tr>
<tr>
<td>Glycophorin B</td>
<td>7.7</td>
<td>170-250</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycophorin D</td>
<td>11.5</td>
<td>82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>MW</td>
<td>Amount</td>
<td>Complexation</td>
<td>EC</td>
<td>Notes</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------</td>
<td>--------</td>
<td>--------------</td>
<td>-----</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Kell</td>
<td>82.8</td>
<td>3-18</td>
<td>Heterodimer with Kx</td>
<td>&lt;1</td>
<td>The Kell-Kx complex may reside in the actin-linked band 3 complex. Kell carries the Kell blood group antigen. Kx probably has an undiscovered transport function.</td>
</tr>
<tr>
<td>Kx</td>
<td>50.9</td>
<td></td>
<td>Heterodimer with Kell</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>Lu/BCAM</td>
<td>64.3</td>
<td>1.5-8</td>
<td>Unknown</td>
<td></td>
<td>Adhesion molecule that carries the Lutheran (Lu) blood group antigen. Binds to α-spectrin.</td>
</tr>
<tr>
<td>LW glycoprotein/ICAM4</td>
<td>27.0</td>
<td>3-5</td>
<td>Monomer</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Intracellular adhesion molecule that carries the Landsteiner-Wiener (LW) blood group antigen. Interacts with RhAG/RhD/RhCE and probably resides in the ankyrin-linked band 3 complex.</td>
</tr>
<tr>
<td>p55/MPP1</td>
<td>52.3</td>
<td>80</td>
<td>Dimer?</td>
<td>1</td>
<td>Links actin junctional complex to membrane via interactions with some protein 4.1R and GPC/D molecules. Heavily palmitoylated. May contribute to organization of lipid rafts.</td>
</tr>
<tr>
<td>Peroxiredoxin 2</td>
<td>21.8</td>
<td>200</td>
<td>Dimer or decamer</td>
<td>?</td>
<td>Abundant antioxidant protein that is partially linked to band 3 on the membrane. Also binds stomatin and activates the Ca²⁺-dependent K⁺ (Gardos) channel.</td>
</tr>
<tr>
<td>Phosphofructokinase-liver</td>
<td>85.0</td>
<td>6</td>
<td>Heterotetramer</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Forms glycolytic metabolon on band 3 with aldolase, G3PD, enolase, PK and LDH.</td>
</tr>
<tr>
<td>Phosphofructokinase-muscle</td>
<td>85.2</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Protein 4.1R</td>
<td>66.4</td>
<td>~240</td>
<td>Monomer</td>
<td>6</td>
<td>Binds to both β-spectrin and actin and greatly strengthens the spectrin-actin interaction. Links actin to the membrane via interactions with GPC/D, p55 and band 3. Competes with ankyrin for binding to band 3.</td>
</tr>
<tr>
<td>Protein 4.2</td>
<td>79.8/76.9</td>
<td>~280</td>
<td>Monomer?</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Binds to band 3, ankyrin and CD47 in the ankyrin-linked band 3 complex. Also binds to the EF hand domain of α-spectrin.</td>
</tr>
<tr>
<td>Protein</td>
<td>M.Wt</td>
<td>M.C.</td>
<td>Description</td>
<td>Copies</td>
<td>Comments</td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>------</td>
<td>--------------------------------------------------</td>
<td>--------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>RhAG</td>
<td>44.2</td>
<td>100-200</td>
<td>Heterotrimer of RhAG, RhD &amp; RhCE?</td>
<td>1</td>
<td>The RhAG/RhD/RhCE trimer binds to CD47, LW, ankyrin, CD47 and GPB in the ankyrin-linked band 3 complex. RhAG transports NH₃/NH₄⁺ and maybe CO₂</td>
</tr>
<tr>
<td>RhCE</td>
<td>45.4</td>
<td>100-200</td>
<td>RhAG, RhD &amp; RhCE?</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>RhD</td>
<td>45.1</td>
<td>100-200</td>
<td>RhAG, RhD &amp; RhCE?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Spectrin</td>
<td>280.0</td>
<td>242</td>
<td>Heterodimer/tetramer/oligomer</td>
<td>2</td>
<td>Crosslinks actin filaments into a hexagonal lattice at the tail end with the help of protein 4.1R, adducin, and dematin. Binds protein 4.2 via the α-spectrin EF hand domain. Self-associates into tetramers and oligomers and binds to ankyrin-1 and Lu/BCAM at the head end.</td>
</tr>
<tr>
<td>β-Spectrin</td>
<td>246.4</td>
<td>242</td>
<td>Monomer</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Stomatin</td>
<td>31.7</td>
<td>200-400?</td>
<td>Oligomer (9-12)?</td>
<td>?</td>
<td>Binds to Glut1 and converts it into an ascorbic acid transporter. Also binds band 3, adducin and peroxiredoxin. Located in lipid rafts.</td>
</tr>
<tr>
<td>Tropomodulin 1</td>
<td>40.6</td>
<td>30</td>
<td>Monomer</td>
<td>1</td>
<td>Caps slow-growing (pointed) end of actin. Binds tropomyosin, which strengthens capping.</td>
</tr>
<tr>
<td>Tropomyosin 1 (αTM)</td>
<td>32.9</td>
<td>140†</td>
<td>Homodimer</td>
<td>2</td>
<td>Binds to and stabilizes actin filaments in a Mg²⁺-dependent manner. May help specify actin filament lengths in the red cell.</td>
</tr>
<tr>
<td>Tropomyosin 3 (γTM)</td>
<td>32.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Size of the mature protein chain without any signal peptide, propeptides or initiator methionine, but not including posttranslational modifications.

† References for the numbers of copies of each protein are listed in the footnotes to Table 2 in reference 6. It must be emphasized that the numbers of many red cell membrane proteins are roughly estimated but not accurately measured, which affects the estimates of the composition of the ankyrin complex and actin junctional complex.

‡ The unit cell is defined in Figure 8.

§ Data shown are for full length ankyrin (band 2.1), the major isoform, except for the number of copies/cell, which includes the smaller isoforms: bands 2.2, 2.3 and 2.6.

Abbreviations: CAII, carbonic anhydrase-II; CAIV, carbonic anhydrase-IV; G3PD, glyceraldehyde-3-phosphate dehydrogenase; Glut1, glucose transporter-1; GPA, glycophorin A; GPB, glycophorin B; GPC/D, glycophorins C and D; LDH, lactic dehydrogenase; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; RhAG, Rh-associated glycoprotein.
### Table 2. Some Unanswered Questions

<table>
<thead>
<tr>
<th>Category</th>
<th>Question</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td>What are the amounts and stoichiometries of the membrane proteins (only a few are accurately measured)?</td>
</tr>
<tr>
<td><strong>Spectrin</strong></td>
<td>What is the structure of spectrin in vivo? How does it change when the membrane is deformed?</td>
</tr>
<tr>
<td></td>
<td>How does spectrin bind to the actin protofilament? What is the stoichiometry? Where do the CH1 and CH2 domains and protein 4.1R bind on actin? Is there more than one binding conformation? If so, how do these differ functionally and how are they regulated?</td>
</tr>
<tr>
<td></td>
<td>Why is the EF hand domain essential for normal spectrin-actin binding? How does it work?</td>
</tr>
<tr>
<td></td>
<td>How do spectrin molecules, which lie in a plane, accommodate different orientations of their binding sites on the helical actin protofilament?</td>
</tr>
<tr>
<td></td>
<td>Higher order spectrin oligomers (hexamers, octamers, etc) also serve as molecular junctions in the skeleton. Are there significant numbers of these and do they have a unique functional role?</td>
</tr>
<tr>
<td></td>
<td>If the available spectrin repeat structures are correct and there is a continuous alpha helix across the inter-repeat junction, how is spectrin flexibility achieved?</td>
</tr>
<tr>
<td></td>
<td>Why are the same repeats in different spectrins so similar? Do they have binding or mechanical functions that we don’t yet know about?</td>
</tr>
<tr>
<td></td>
<td>What binds to the spectrin SH3 domain? What are the consequences?</td>
</tr>
<tr>
<td></td>
<td>There is evidence that some of the spectrins in mature red cells contain the muscle isoform of β1-spectrin, which has a lipid-interacting pleckstrin homology domain. Is this true, and if so, what is its function?</td>
</tr>
<tr>
<td><strong>Actin</strong></td>
<td>How are actin protofilaments formed? How do they achieve their uniform size? Do formins play a role?</td>
</tr>
<tr>
<td></td>
<td>Are the actin protofilaments stable or do they turnover, as the presence of a critical concentration of G-actin in the red cell cytoplasm suggests? If so, how is this regulated?</td>
</tr>
<tr>
<td></td>
<td>How are the various proteins arranged on the actin protofilament? Is the arrangement the same in all the protofilaments? If there are open binding sites on actin, do molecules like spectrin move from site to site with membrane deformation?</td>
</tr>
<tr>
<td></td>
<td>Do red cell myosin and caldesmon have a function in the membrane of mature red cells?</td>
</tr>
<tr>
<td><strong>Ankyrin</strong></td>
<td>Does ankyrin bind a band 3 tetramer or two dimers? If a tetramer, is it preformed before binding or do two dimers bind and convert to a tetramer on ankyrin. What is the structure of the band 3 tetramer-ankyrin complex?</td>
</tr>
<tr>
<td></td>
<td>What is the structure of the ankyrin molecule? How do the various domains relate to each other spatially?</td>
</tr>
<tr>
<td></td>
<td>Where do protein 4.2 and RhAG bind?</td>
</tr>
<tr>
<td></td>
<td>What is the function of the death domain in ankyrin?</td>
</tr>
<tr>
<td></td>
<td>What are the functions of the many ankyrin spliceoforms?</td>
</tr>
</tbody>
</table>
| **Band 3** | • What is the overall structure of band 3?  
• Where do protein 4.2 and adducin bind on band 3?  
• Since the numbers of integral membrane proteins vary by orders of magnitude (Table 1), the band 3 multiprotein complexes must vary in composition. How many such complexes are there? Do they have specific, stable compositions or are they in equilibrium and continually changing their composition? Are the sub-stoichiometric proteins (e.g., CD44, CD47, LW, Kx/Kell, DARC) uniformly distributed or localized to lipid rafts or other membrane lipid or protein subdomains?  
• How are the proteins in the band 3 multiprotein complexes arranged relative to each other?  
• Do protein interactions within the band 3 multiprotein complexes allosterically affect the interactions or functions of other proteins in the complexes?  
• Does protein 4.1R bind to band 3 in vivo? If so, does band 3 bind to each of the spectrin-actin-4.1R complexes or only to a subset, such as the subset that interacts with adducin (Figure 8)?  
• Are any band 3 molecules “unbound” (i.e., not attached to the membrane skeleton directly or indirectly). If so, how many? Are they also part of multiprotein complexes? Do they have a unique function? |
| **Glycolytic Metabolon** | • The enzymes that comprise the proposed glycolytic metabolon vary in concentration by orders of magnitude. Are the estimates correct? If so, do some complexes lack rare components (but then how would they function?) or are they gigantic so as to include at least one copy of each enzyme? Are other enzymes in the glycolytic pathway part of the complex?  
• How is the glycolytic metabolon regulated in vivo? What is the purpose of activating glycolysis by deoxyhemoglobin? Do products of glycolysis, such as ATP cause vasodilation, directly or indirectly? Does nitric oxide play a role? Is the rate of dissociation and activation of the glycolytic metabolon rapid enough to be relevant during normal circulatory transits? |
| **Protein 4.1R** | • What is the structure of intact protein 4.1R and the spectrin-actin-4.1R complex?  
• Calmodulin binds to and regulates protein 4.1R. The EF hand domain is a calmodulin-like structure that resides near 4.1R in the actin junctional complex. In addition, the EF domain binds calmodulin. Does 4.1R bind to the EF domain or do they bind each other through calmodulin? If so is this a regulatory interaction?  
• Does phosphatidyl-4,5-bisphosphate regulate protein 4.1R binding to actin or other proteins in vivo?  
• Since there is insufficient protein p55/MPP to make a 1:1:1 molar complex with protein 4.1R and glycophorin C/D, how is the complex constructed? |
<p>| <strong>Protein 4.2</strong> | • What is the function of protein 4.2 when attached to the spectrin EF hand domain? Does it actually occupy the domain in vivo? If so, does it bind to protein 4.1R? |</p>
<table>
<thead>
<tr>
<th>Section</th>
<th>Questions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adducin</td>
<td>• What is the function of the ATP-binding site on protein 4.2? &lt;br&gt; • Is adducin a dimer or tetramer? &lt;br&gt; • What is the structure of adducin? How does the C-terminal tail relate to the head end? &lt;br&gt; • Band 3, spectrin and actin all bind to the C-terminal tail. What is the structure of this complex? How are the multiple interactions regulated? Can they all occur at once on the same molecule or do some interactions interfere with others? When adducin binds spectrin and actin does it also interact with nearby proteins 4.1R or 4.2? Does adducin bind band 3 dimers or tetramers and does it bind one or two molecules of each?</td>
</tr>
<tr>
<td>Dematin</td>
<td>• How does dematin contribute to spectrin binding in vivo? &lt;br&gt; • Is dematin’s ability to bundle actin filaments important in the mature red cell? &lt;br&gt; • Where does dematin bind relative to spectrin and adducin on the actin protofilament? Does the number of dematins per protofilament vary?</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>• Do the red cell isoforms of tropomyosin determine the unique structure of the short actin protofilaments? Do specific actin-nucleating formins play a role in this process?</td>
</tr>
<tr>
<td>Post-translational Modifications</td>
<td>• What do post-translational modifications like phosphorylation, palmitoylation, myristoylation, hydroxylation, methylation, glycation and ubiquitination do? Are the effects important in vivo? &lt;br&gt; • How are the multiple effects of Ca(^{2+}) and calmodulin integrated in vivo?</td>
</tr>
<tr>
<td>Dynamics</td>
<td>• Do the actin junctional complex and ankyrin complex contact each other in vivo? Do proteins like band 3, proteins 4.1R and 4.2, and adducin, which have potential binding partners in both complexes, sometimes switch allegiances? If so, is this be a regulatory process? &lt;br&gt; • How labile are the individual bonds in the membrane skeleton? Which bonds, other than the spectrin dimer-tetramer interaction, break when the skeleton is deformed? Are the rates of dissociation and reassociation such that the bonds dissociate during red cell deformation in the circulation? &lt;br&gt; • How is the skeleton disassembled and reassembled during invasion by malaria and other parasites?</td>
</tr>
<tr>
<td>Lipids</td>
<td>• What are the interactions between the membrane skeleton and the overlying lipids? Do these interactions have regulatory as well as structural functions? Does the membrane skeleton vary in unique regions of the lipid bilayer, such as lipid rafts?</td>
</tr>
</tbody>
</table>
Legends

Figure 1. The first model of the red cell membrane skeleton, published 36 years ago. (Reprinted from Lux SE\textsuperscript{4} with permission.)

Figure 2. Current model of the red cell membrane. Most of the known protein contacts are shown, but the relative positions of the proteins to each other within the various complexes are mostly not known. The major proteins are drawn roughly to scale but the shapes are mostly imaginary. Roughly 40% of the band 3 molecules are tetramers in a complex with ankyrin and other integral proteins near the spectrin self-association site (“Ankyrin Complex”). A roughly similar fraction of the band 3 molecules, probably dimers, are located near the spectrin-actin junction and bind to spectrin via protein 4.1R, protein 4.2 and adducin (“Actin Junctional Complex”). As described later in the text, it is likely these two complexes, with their associated proteins, are large enough that they sometimes contact each other. The remaining band 3 dimers float untethered within the lipid bilayer (“Unbound Band 3”). The actin protofilament lies parallel to the membrane. The complexes of proteins associated with band 3 are not constant—some proteins are present in much smaller numbers than others (e.g., Kell, Kx, CD44, CD47, DARC/Duffy, LW, PFK and aldolase). The amounts of p55, adducin and dematin are also insufficient to interact with all the spectrin-4.1-actin complexes. As a consequence, the ankyrin and actin junctional complexes must vary in composition and mobility. For visual clarity, peroxiredoxin-2 is shown attached just to unbound band 3 but there is no evidence for this selectivity. Ank, ankyrin; CH1 and CH2, actin binding domains of β-spectrin; EF, Ca\textsuperscript{2+}-binding EF hand domain of α-spectrin; GEC, glycolytic enzyme complex (G3PD, PFK, LDH, PK, aldolase and enolase); GPA, glycophorin A; GPB, glycophorin B; GPC, glycophorin C; Prx2, peroxiredoxin-2; RhAG, Rh-associated glycoprotein. (Professional illustration by Somersault18:24. Adapted from Korsgren C, Peters LL, Lux SE,\textsuperscript{5} with permission.)
Figure 3. Spectrin. (A) Organization of erythrocyte spectrin and the dimer-tetramer equilibrium. Spectrin is a long, flexible, worm-like protein composed of two chains (α- and β-spectrin). Each chain contains a tandem array of ~5.0 nm, ~106-amino acid, triple helical spectrin-type repeats with specialized functional domains for self-association and ankyrin-1 binding at the “head” end, and for binding to actin, protein 4.1R and other associated proteins at the “tail” end. Each spectrin repeat is formed by three α-helices (A, B & C) with short connecting loops that are folded like a flattened Z into a triple helical bundle.\(^{12}\) α-Spectrin contains 21 numbered repeats (α1-α21), plus a partial repeat (α0) at the N-terminus that contains a single C-helix. One of the 21 is really an SH3 domain (α10), but is numbered as a repeat by convention. β-Spectrin contains 16 true repeats (β1-β16) plus a partial repeat (β17) at the C-terminus that contains just the A and B helices. Note that for the spectrin αβ-dimer to convert to the α\(^2\)β\(^2\)-tetramer it must first cleave the internal linkage between the partial spectrin repeats α0 and β17 and then unfold (open dimer).\(^{13}\) This is the rate-limiting step in the dimer-tetramer equilibrium. Dimer-tetramer self-association occurs at the “head” end of the spectrin dimer where the adhesion protein Lu/BCAM also attaches.\(^{14}\) Ankyrin-1 binds nearby to spectrin repeats β14-β15.\(^{15}\) These two reactions cooperate: ankyrin-binding favors spectrin tetramer formation and vice-versa.\(^{16}\) The isolated α- and β-spectrin chains join to form spectrin heterodimers at a nucleation site near the “tail” end of spectrin (repeats α21 pairs with β-1 and α-20 with β-2) and then zip together in a cooperative manner.\(^{17}\) Actin and protein 4.1R bind to CH domains at the N-terminal end of β-spectrin, just beyond the nucleation site.\(^{18}\) Binding to the CH2 domain is activated by phosphatidylinositol-4,5-bisphosphate (PIP2).\(^{18}\) Adducin binds in the same region.\(^{19}\) Protein 4.2 and calcium bind to a neighboring EF-hand domain on α-spectrin.\(^{5}\) Both the CH and EF-hand domains are needed for full actin binding.\(^{20,21}\) PS denotes spectrin repeats that bind phosphatidyl serine.\(^{22}\) Blue stars mark repeats that are relatively unstable at physiological temperatures.\(^{23}\) (B) Structure of spectrin repeats β8 and β9 (Protein Data Bank: 1S35). Note that each repeat is formed by three α-helices (A, B & C) in a Z-configuration. Note also that helix C in β8 and helix A in β9 form a continuous, α-helix that spans the junction between the repeats. (C) Hypothetical model of the tail end of spectrin based on recent structures of α-actinin.\(^{24,25}\) Note that the first actin binding
domain (CH1) binds to F-actin in an extended (open) conformation. The intimate relationship between the EF hand and CH domains and recent evidence that the EF domain is required for optimal spectrin-actin binding suggest the EF hands regulate actin binding.

**Figure 4. Protein 4.1R.** (A) Domain map of protein 4.1R and the location of erythrocite binding partners. (B) Structure of the protein 4.1R membrane binding domain (MBD). Subdomains where specific proteins bind are colored and labelled. (C) Schematic representation of the protein 4.1R spectrin-actin binding domain (SABD, blue). Two spectrin binding regions straddle an actin binding peptide. In this hypothetical model, each of the spectrin binding regions is assumed to interact with a different one of the two calponin homology (CH) domains of β-spectrin and both CH domains are assumed to interact with actin. Neither of these things has been proved. *(Panel B adapted from Han BG, et al. with permission.)*

**Figure 5. Negatively-stained electron micrographs of red cell membrane skeletons and spectrin.** (A) A membrane skeleton that was stretched during preparation. Note the pseudohexagonal organization of the skeleton and the location of various proteins. *Inset, bottom left.* Example of a 37 nm long F-actin protofilament. Sp4, spectrin tetramer; Sp6, spectrin hexamer. (B) Skeleton in situ in a red cell prepared by a minimally perturbing quick-freeze, deep etch, rotary replication procedure. The dense network of filaments average 29 ± 9 nm between intersections. (C) and (D) Spectrin from partially stretched skeletons. Some of the spectrin molecules show a helical periodic substructure, as noted by the region between the arrowheads in C or by the single arrowhead in D. (E) Right handed double-helical models of spectrin periodicity obtained from the experiments in D by visual filtering of the periodic regions from multiple spectrin molecules. In this model, the spring-like spectrins extend and contract by varying their pitch and diameter. Native spectrin tetramer has approximately 10 turns with a pitch of about 7 nm (70 Å) and a diameter of about 5.9 nm (59 Å), *(A) Reprinted from Liu SC et al. Inset from Byers T and Branton D. (B) From*
Figure 6. Ankyrin. (A) Schematic of erythroid ankyrin (Ank1 or AnkR) structure. The Membrane Domain contains 24 ankyrin repeats, grouped functionally into four subdomains of six repeats. There are two binding sites for band 3, one involving repeats 7 to 12 (D2, domain 2), and one involving repeats 13 to 24 (D34). The ankyrin binding loops on band 3 are predicted to interact with ankyrin repeats 19 and 20 (light blue) of D4. The interaction site within D2 is not known. The Spectrin Domain contains three subdomains of which ZU5A (light blue) contains the binding site for spectrin. The C-Terminal (Regulatory) Domain is thought to modulate the binding functions of the other two domains. It exists in numerous spliced variants of mostly unknown function. The function of the conserved death domain (DD) is also a mystery. (B) Hypothetical model of the interaction between the membrane domain of ankyrin (green) and a band 3 tetramer (red, blue, cyan and purple subunits). Note how the ankyrin repeats form a large (9 nm diameter) twisted helical spiral. In this deduced model, the concave surface of the D34 region interacts with the red subunit in one band 3 dimer. One subunit in the second band 3 dimer contacts the concave surface of the D2 region, which contains a second band 3 binding site. (C) Spectrin-ankyrin interaction. Note how ZU5-A, the spectrin-binding subdomain within ankyrin, binds in the notch created by the sharp angle between spectrin repeats $\beta_{14}$ and $\beta_{15}$. (Panel B from Michaely P, et al and panel C from Ipsaro JJ and Mondragón A with permission.)

Figure 7. Band 3. Organizational model of human erythrocyte band 3 (anion exchange protein). The protein contains two structurally and functionally distinct domains: a cytoplasmic binding domain (amino acids (aa) 1-359) and a transmembrane domain (aa 360-911) that forms the anion exchange channel. Cytoplasmic Domain: The glycolytic enzymes phosphofructokinase (PFK), aldolase, and glyceraldehyde-3-phosphate (G3PD) bind to amino acids 1-23 at the N-terminus of band 3 and contact amino acids 356-384, which are
nearby in the folded protein. The enzymes are inactive when bound\textsuperscript{79} but they are displaced and activated by deoxyhemoglobin, which also binds to the N-terminus,\textsuperscript{80} or by phosphorylation of two tyrosines within the binding sites.\textsuperscript{79} Enolase, pyruvate kinase (PK) and lactic dehydrogenase (LDH) also localize to the membrane and are displaced by deoxyhemoglobin, but \textit{don’t} bind to band 3.\textsuperscript{79} This suggests that many of the enzymes in the glycolytic pathway form a functional complex (or ‘metabolon’) that efficiently generates ATP, particularly under hypoxic conditions. Ankyrin also interacts with the N-terminus,\textsuperscript{81} but the main ankyrin sites are aa 63-73 and 175-185, which loop out from the surface.\textsuperscript{70} Protein 4.1 binds to two sites with the (I/L)RRRY motif, near the end of the domain.\textsuperscript{82} Ankyrin and protein 4.1R inhibit each other’s binding. The binding sites for protein 4.2 and adducin have not been identified. \textit{Membrane Domain:} The best current model\textsuperscript{83} for the anion exchange channel is based on the structure of the ClC bacterial chloride channel. The intra- and trans-membrane helices are lettered as they are in the ClC channel. A complex carbohydrate structure is attached to Asn 642. Carbonic anhydrase IV binds to the extracellular loop of band 3 between helices I and J,\textsuperscript{84} and CAII may bind to the C-terminal segment.\textsuperscript{85} Both are perfectly positioned to create HCO\textsubscript{3}\textsuperscript{-} from CO\textsubscript{2} and shuttle the ions to or from the plasma through band 3.

\textbf{Figure 8. Model of the unit cell of the membrane skeleton.} (A) The membrane skeleton is a quasi-hexagonal lattice centered around \textasciitilde40,000 F-actin protofilaments. Each 37 nm, double helical protofilament is capped by adducin (A) and tropomodulin (T) and contains about 14 G-actin subunits. The red cell surface area is about 140 \textmu m\textsuperscript{2} so each hexagonal unit cell is 3500 nm\textsuperscript{2} and the average length of a spectrin dimer is about 32 nm, which is one-third it’s length when fully stretched. Available data suggests the spectrin chains are coiled about each other, and expand and contract by changing their pitch and diameter\textsuperscript{27} (Figure 5E). They are shown here as straight, though they are sometimes kinked.\textsuperscript{30} Spectrin oligomers are not shown in this model but exist in vivo. In addition, the skeleton is not as regularly arrayed in vivo as in this averaged model. (B) A recent model of the ankyrin complex (\textit{green ovals}) assembled from known structures of the proteins by Burton and Bruce\textsuperscript{7} has a cross-sectional area of 17.5 nm x 14 nm, assuming the proteins are
closely packed. They estimate the cross-sectional area of the actin junctional complex is about 20 nm x 17 nm if it only contains a single band 3 dimer (small tan ovals). Such a complex would presumably lie toward the end of the actin protofilament where adducin resides. This is shown in the two unit cells on the right.

Alternatively, if the actin junctional complex contains six band 3 dimers interacting with all six 4.1R proteins, it would probably be more centered and roughly approximate the area of the large tan oval in the unit cell on the left. Recent data favor the larger arrangement, but in either case it is likely the ankyrin complex and actin junctional complex would be close to each other and probably often touch, particularly during red cell deformation. Band 3 dimers that are not associated with either the ankyrin or actin junctional complexes (small blue ovals) diffuse in the lipid bilayer within the spectrin corrals. There will be three to eight of these per unit cell depending on the number of band 3’s in the actin junctional complex. (From Lux SE with permission.)
Figure 4

A

<table>
<thead>
<tr>
<th>Protein:</th>
</tr>
</thead>
<tbody>
<tr>
<td>30K</td>
</tr>
<tr>
<td>16K</td>
</tr>
<tr>
<td>10K</td>
</tr>
<tr>
<td>22-24K</td>
</tr>
</tbody>
</table>

Binding sites:
- Glycophorin C
- Calmodulin
- Protein p55
- Band 3
- CD44
- PS/PIP₂

SABD
- Spectrin/Actin

B

CaM
- Ca²⁺-sensitive
- Ca²⁺-insensitive

Band 3

GPC

C

4.1R

β-Spectrin

Actin
Anatomy of the red cell membrane skeleton: unanswered questions

Samuel E. Lux IV