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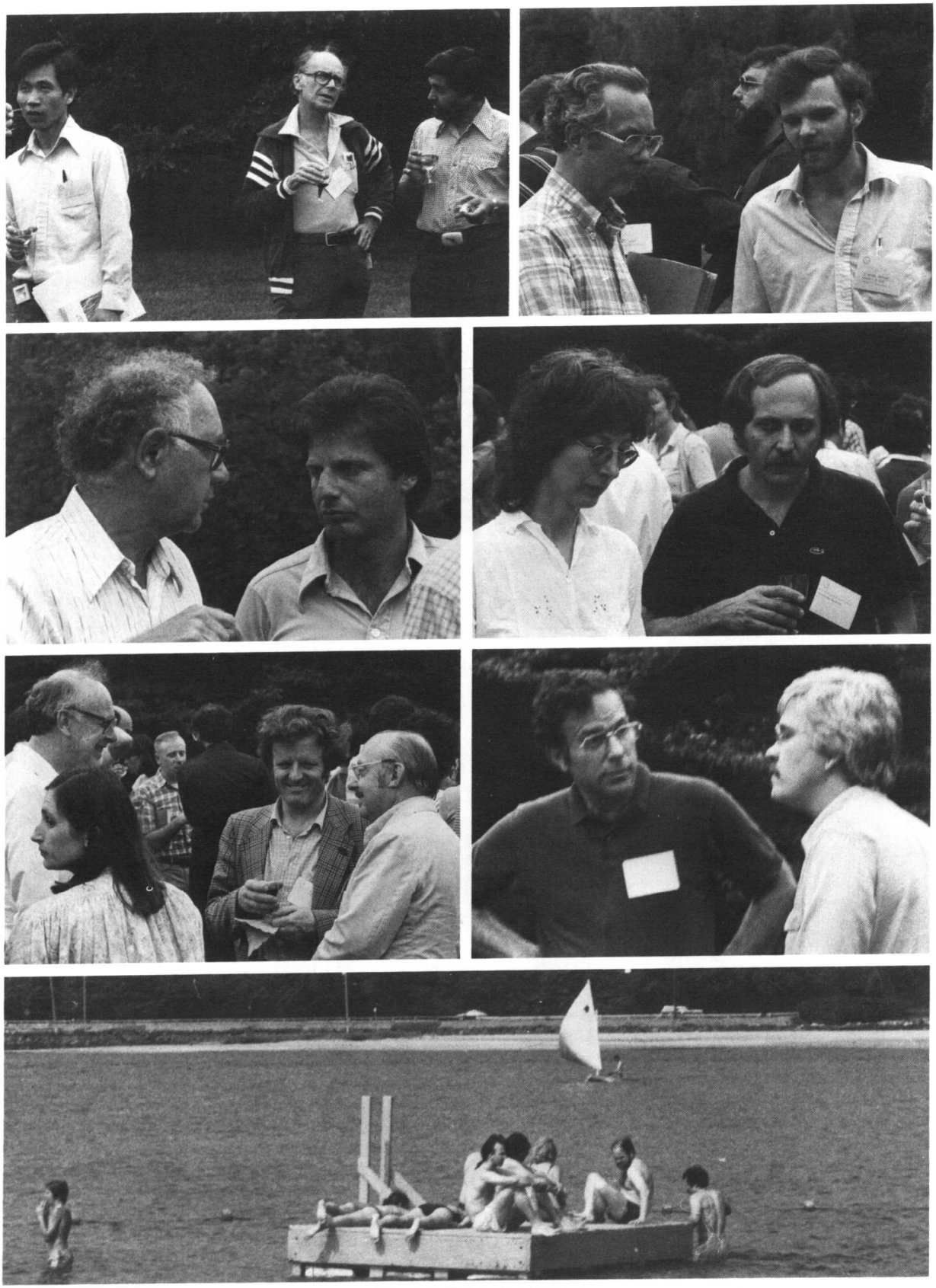

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Membrane Cytoskeletal Interactions in the Human Erythrocyte

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When an erythrocyte membrane is extracted with a nonionic detergent such as Triton X-100, an interconnected reticulum or shell remains (Yu et al. 1973). This reticulum constitutes the membrane skeleton or erythrocyte cytoskeleton. Interest in the erythrocyte membrane and its associated proteins has been heightened by the realization that membrane skeletons exist in other cell types (Ben-Ze'ev et al. 1979; Luna et al. 1981; Mescher et al. 1981) and that the transcellular cytoskeletal structures found in all eukaryotic cells contain some of the same elements (actin, actin-binding proteins, and cytoskeletal anchorage sites) present in the erythrocyte cytoskeleton. The results of biochemical and electron microscopic investigations have now indicated the major molecular features of the erythrocyte cytoskeleton (Fig. 1). Although it is doubtful that these features per se can serve as a model for all membrane skeletons or cytoskeletons, many of the approaches and lessons that have emerged from studying the erythrocyte cytoskeleton will continue to serve as guidelines during the analysis of more complex cytoskeletal structures.

In this laboratory, strategies for analyzing the erythrocyte cytoskeleton have involved selective elution and purification of membrane components, assays of binding between eluted components and membrane vesicles, association assays in solution, and visualization of site-specific binding by electron microscopy. Here, I summarize how these strategies have served to develop our current understanding of the erythrocyte cytoskeleton (for details, see Branton et al. 1981), and I discuss why these strategies are limited to providing insight into the first-order interactions of individual molecules but few predictions about the next hierarchical levels of structure involving the overall organization of a cytoskeleton.

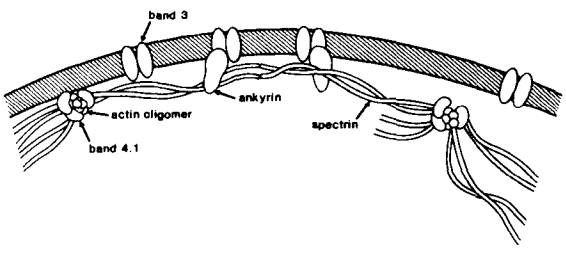

Figure 1. Drawing showing the general structure and linkages of the major polypeptides of the erythrocyte cytoskeleton (Branton et al. 1981).

Membrane Binding Studies

The first evidence for a specific association between the membrane and a cytoskeletal protein came from studies (Bennett and Branton 1977) in which purified spectrin, one of the major cytoskeletal components, was reassociated with spectrin-depleted membranes (Fig. 2). Once this assay, which quantified the reassociation of cytoskeletal and membrane components, was available, it became possible to identify the component that was binding the spectrin. The crucial step that led to this identification was the isolation from spectrin-depleted inside-out membranes of a chymotryptic fragment capable of competing with inside-out vesicles for the rebinding of spectrin (Bennett 1978). Using antibodies to this fragment, Bennett and Stenbuck (1979a) were able to show that it was derived from band 2.1, a polypeptide they named ankyrin.

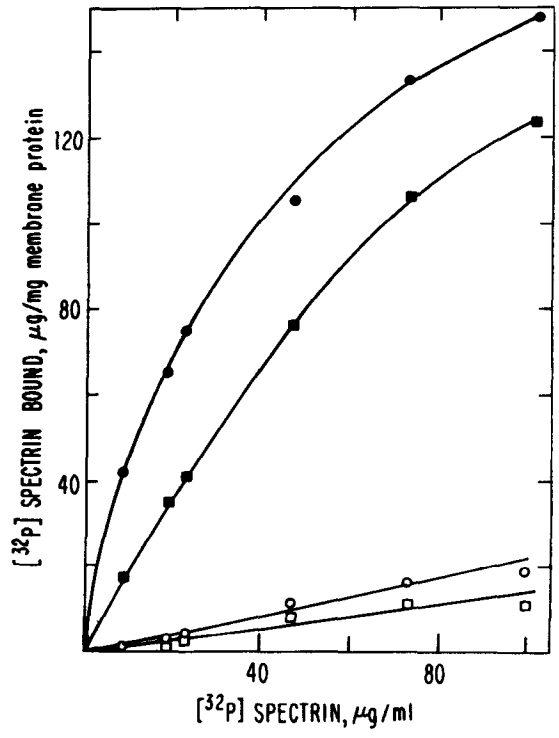

Figure 2. The binding of spectrin to erythrocyte membranes. The amount of [^{32}P]spectrin which binds to spectrin-depleted inside-out vesicles (●,■) or right-side-out vesicles (○,□) is plotted as a function of spectrin concentration at pH 6.6 (●,○) and pH 7.6 (■,○) (Bennett and Branton 1977).

Using a different approach, Luna et al. (1979) and Yu and Goodman (1979) showed by two-dimensional peptide mapping that the fragment was derived from band 2.1 and the sequence-related group of polypeptides designated as bands 2.2–2.6. Bands 2.2–2.6 are not simply breakdown products formed from band 2.1 during ghost production (Siegel et al. 1980), but the similarities between these polypeptides, band 2.1, and the fragment suggest that all are spectrin-binding proteins. They have been referred to as syndeins (Yu and Goodman 1979), but the name ankyrin has found widespread acceptance.

Having identified ankyrin as a crucial spectrin-binding site, it was important to determine how ankyrin itself, which could be eluted from the membrane with high-salt treatments, was anchored. The first clue that band 3, a major transmembrane protein and the erythrocyte anion transporter, was the binding site for ankyrin came from detergent solubilization studies that showed band 3 together with ankyrin and band 4.2 in detergent complexes (Bennett and Stenbuck 1979b). Subsequent steps to elucidate the role of band 3 as the membrane binding site for ankyrin paralleled the approaches used in studying the association of spectrin with membranes: The reassociation of purified ankyrin with ankyrin-depleted membranes was quantified, and competition assays using proteolytic fragments of putative binding sites were performed (Bennett and Stenbuck 1980; Hargreaves et al. 1980). Although these studies made it clear that band 3 is the integral protein which is the receptor that binds ankyrin to the membrane, the number of ankyrin molecules in an erythrocyte is far less than the number of band-3 polypeptides. The significance of this discrepancy remains to be clarified.

Although the association of spectrin with the membrane is well understood, the membrane association of actin is just beginning to be characterized. It has been shown that binding of preformed F-actin to spectrin- and actin-depleted inside-out vesicles is promoted by prior reconstitution of the vesicles with purified spectrin (Cohen and Foley 1980; Fowler et al. 1981). Binding of F-actin to spectrin-reconstituted vesicles is further enhanced by addition of purified band 4.1, another cytoskeletal polypeptide. Furthermore, spectrin exists as a heterodimer and as a tetramer, and spectrin tetramers are twice as effective as spectrin dimers (per mole) at stimulating actin binding (Cohen and Foley 1980). In vivo, spectrin tetramers far outnumber the heterodimers (Goodman and Weidner 1980; Liu and Palek 1980).

The binding of F-actin to membranes has also been measured by a low-shear viscometric technique (Fowler et al. 1981). The rationale for these measurements is that if membranes have binding sites for F-actin, they will be able to cross-link and increase the viscosity of an actin preparation. Indeed, spectrin- and actin-depleted inside-out vesicles reconstituted with purified spectrin induce large increases in the viscosity of actin, whereas spectrin alone or inside-out vesicles not reconstituted with spectrin have no effect on the viscosity of actin (Fig. 3). These and other results using low-shear viscometry confirm that spectrin provides a crucial binding site that attaches actin filaments to the erythrocyte membrane.

Association Assays in Solution

Reassociation assays using selectively depleted membranes provided the first clues that identified the binding interactions of cytoskeletal proteins. Many of these interactions have now been confirmed and extended by solution studies using purified polypeptides. By using purified components in the absence of membranes, potentially complicating effects of multiple protein-protein or protein-lipid interactions can be avoided.

For example, solution studies with ankyrin and spectrin or with spectrin and band 4.1 (Tyler et al. 1980) allowed separate determinations of the dissociation constants and stoichiometric values for each of these pairs. Because band 4.1 remained associated with the spectrin-depleted inside-out vesicles used in the spectrin-membrane binding studies (Bennett and Branton 1977), its contribution to spectrin binding was hard to evaluate using membrane binding studies alone. By measuring the associations of the purified components, it became clear that ankyrin and spectrin and spectrin and band 4.1 interactions are distinct associations, each of which can occur in the absence of the other. Solution studies have also been useful in confirming

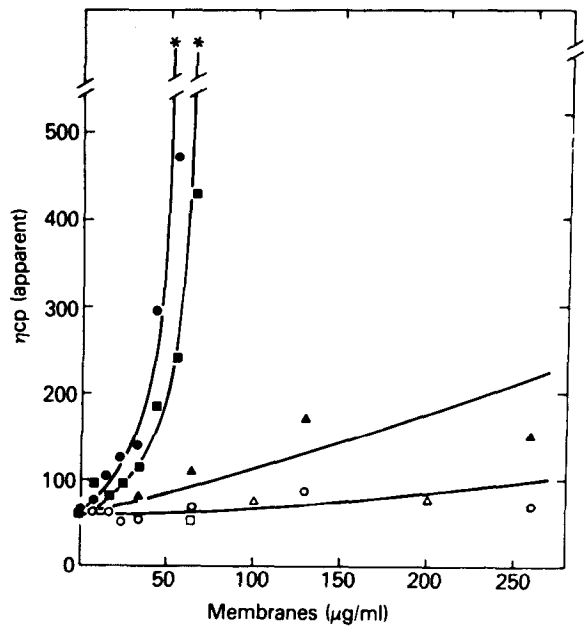

Figure 3. Viscosity changes as a measure of actin-membrane interaction. Changes of viscosity in a preparation of actin mixed with varying concentrations of spectrin-depleted inside-out vesicles reconstituted with no spectrin (○), 2 μg/ml spectrin (▲), 5 μg/ml spectrin (■), 30 μg/ml spectrin (●), or 5 μg/ml heat-treated spectrin (□). Controls using intact, right-side-out ghosts (△) are also shown (Fowler et al. 1981).

the association between spectrin and ankyrin (Tyler et al. 1979), between ankyrin and the cytoplasmic domain of band 3 (Bennett and Stenbuck 1980), between spectrin and actin (Brenner and Korn 1979; Ungewickell et al. 1979; Cohen and Korsgren 1981), and between spectrin, band 4.1, and actin in ternary complexes (Ungewickell et al. 1979: Cohen and Korsgren 1981).

Structural Analysis

Because a membrane skeleton or cytoskeleton is defined in terms of its structural role, it is important that the biochemical data showing protein-protein connections be supplemented by studies that provide information about the structure of these interconnecting elements. Direct electron microscopic examination of the interacting molecules was facilitated by examining the purified polypeptides that had been allowed to associate in solution and then mixed with glycerol. When these solutions were sprayed onto mica, dried in vacuo, and rotary-shadowed with heavy metals, the protein-protein interactions were not disturbed and the individual molecular shapes were displayed in a readily interpretable manner (Tyler and Branton 1980).

Electron micrographs of rotary-shadowed spectrin showed the heterodimer as two loosely intertwined, long, flexible strands joined at their ends (Shotton et al. 1979). The heterodimer was approximately 100 nm long, and the tetramer was 200 nm long. These simple electron microscopic observations indicated that the tetramer must be formed by the end-to-end association of two heterodimers. Similar electron micrographs of dried solutions in which two or more of the erythrocyte cytoskeletal proteins were reassociated made it possible to map the location of specific binding sites along the length of the spectrin molecule. Thus, ankyrin binding sites are located approximately 200 Å from the head end of the spectrin heterodimer that participates in tetramer formation (Fig. 4a), and band 4.1 and actin-binding sites are located at the tail end of the heterodimer farthest from the end that participates in tetramer formation (Fig. 4b,c). Long actin filaments, such as those shown in Figure 4c, do not exist in the erythrocyte where short actin oligomers probably predominate (Branton et al. 1981). Electron micrographs show that such oligomers also bind at a site located at the tail ends of the spectrin molecule (Cohen et al. 1980).

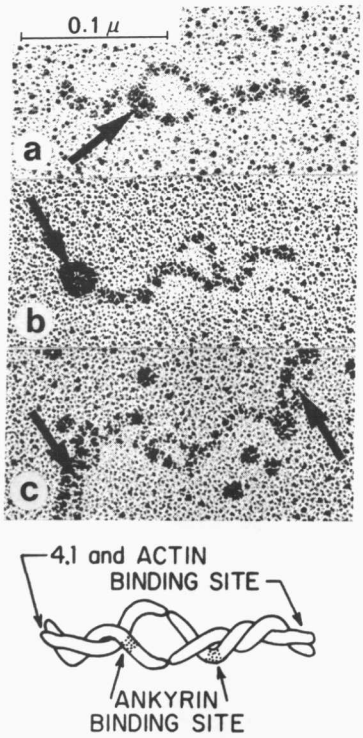

Figure 4. Electron micrographs that localize binding sites of spectrin for ankyrin *(a)*, band 4.1 *(b)*, and actin *(c)*. The binding sites (←) are demonstrated by observing the position of the bound ankyrin molecule *(a)* or actin filament *(c)*, or by observing the bound band-4.1 molecule that has been labeled with ferritin to make it clearly visible *(b)*. *(a,b,c)* Adapted from Tyler et al. (1979), Tyler et al. (1980), and Cohen et al. (1980), respectively.

Membrane-Protein Interactions and the Organization of the Erythrocyte Cytoskeleton

The view of the erythrocyte cytoskeleton that emerges from these biochemical and structural studies is that of a reticulum composed of spectrin tetramers together with band 4.1 cross-linked by their association with short actin oligomers (Fig. 1). This reticulum is bound to the membrane by spectrin-ankyrin and ankyrin–band-3 interactions.

These studies of the protein interactions on the erythrocyte membrane have mapped out some of the major connections that link the proteins to each other. The role of other minor polypeptides, such as band 4.9, and the manner in which the known interactions are regulated remain to be established. But even before we answer these questions, it is useful to ask how the overall reticulum is organized and what the relationship of the individual molecular interactions is to the contours of the membrane and the shape of the cell. The substance of this question is made clear (Fig. 5) if one attempts to extrapolate from the known associations of an individual spectrin tetramer to the unknown arrangements that the many tetramers in one erythrocyte cytoskeleton can assume.

There are several reasons why it is impossible to make many predictions about the overall organization of the erythrocyte cytoskeleton from what we have learned about the first-order interactions of individual molecules: (1) The cytoskeletal reticulum is dominated by an elongated actin-binding protein, spectrin, that is extremely flexible and can assume a wide variety of configurations (Shotton et al. 1979). We do not know the extent to which the spectrin molecule is folded upon itself in vivo. (2) The cytoskeleton is anchored to the membrane at sites whose position is not fixed

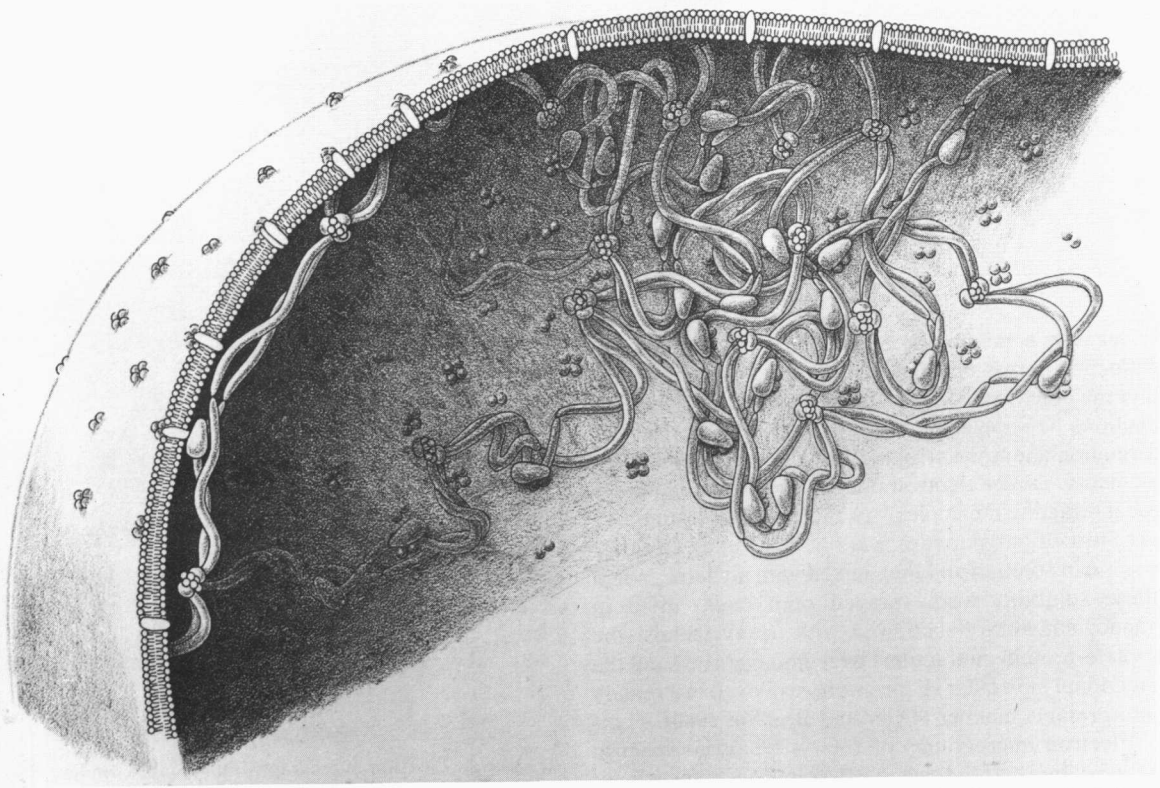

Figure 5. Associations of proteins in the erythrocyte cytoskeleton. An individual spectrin tetramer is shown at the left to illustrate the known first-order interactions discussed in the text. The overall structure of the cytoskeleton is not known. The meshwork at the right is drawn to indicate one of many ways in which the proteins could assemble to approximate the known density of molecules on the membrane (Cohen and Branton 1981).

(Elgsaeter and Branton 1974). It is therefore not known whether neighboring spectrin molecules cross over each other or lie curled next to each other. (3) Both our biochemical and our structural analyses measure relatively high-affinity interactions; but low-affinity interactions may be very important at the very high protein concentrations in the immediate neighborhood of the membrane. Low-affinity interactions could, for example, promote further oligomerization of cytoskeletal components (Morrow and Marchesi 1981). (4) Although it is believed widely that the shape of the red cell is primarily controlled by its cytoskeleton, there is also evidence that the cytoskeleton conforms to shape-determining factors inherent in the membrane bilayer (Steck and Lange 1981). Furthermore, many cellular components, some of which may be important in determining the overall assembly of the cytoskeleton, are lost during erythroid development.

Taken together, these facts make it unlikely that even a detailed description of the interactions which can be probed by binding studies and electron microscopy will make it possible to predict the next hierarchical level of structure involving the organization of the entire cytoskeleton and its role in determining cell shape.

CONCLUSION

The problems we face in the erythrocyte in extending from one level of organization to the next are encountered many times in probing the organization of a eukaryotic cell cytoplasm. It is essential that we understand the molecular interactions to discover the constraints which these interactions place on the next hierarchical level of structure. But it is also clear that all of the rules that dictate cytoplasmic organization will not be found in those relatively high-affinity interactions that are most easily measured and interpreted using current approaches. The challenge is to discover the approaches that will make it possible to understand the logic of more elaborate systems in terms of the rules of molecular interactions.

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