Journal of Cellular Biochemistry 18:49–65 (1982)
Cellular Recognition 513–529

The Molecular Basis for Membrane – Cytoskeleton Association in Human Erythrocytes

Vann Bennett

Department of Cell Biology and Anatomy, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205

Spectrin, the major cytoskeletal protein in erythrocytes, is localized on the inner membrane surface in association with membrane-spanning glycoproteins and with intramembrane particles. The presence of a specific, high-affinity protein binding site for spectrin on the cytoplasmic surface of the membrane has been established by measurement of reassociation of spectrin with spectrin-depleted inside-out vesicles. A 72,000 M_r proteolytic fragment of this attachment protein has been purified, which bound to spectrin in solution and competed for reassociation of spectrin with vesicles. A 215,000 M_r polypeptide has been identified as the precursor of the spectrin-binding fragment. The membrane attachment protein for spectrin was named ankyrin, and has been purified and characterized. Ankyrin has been demonstrated to be tightly associated in detergent extracts of vesicles with band 3, a major membrane-spanning polypeptide, and to bind directly to a proteolytic fragment derived from the cytoplasmic domain of band 3. Ankyrin is thus an example of a protein that directly links a cytoplasmic structural protein to an integral membrane protein. The organization of the erythrocyte membrane has implications for more complex cell types since immunoreactive forms of ankyrin distinct from myosin or filamin have been detected by radioimmunoassay in a variety of cells and tissues. Indirect immunofluorescent staining of cultured cells reveals immunoreactive forms of ankyrin in a cytoplasmic meshwork and in a punctate distribution over nuclei. The staining changes dramatically during mitosis, with concentration of stain at the spindle poles in metaphase and intense staining of the cleavage furrow during cytokinesis.

Key words: erythrocyte membrane, cytoskeleton, membrane protein, microtubule-associated protein, hemolytic anemia, hereditary spherocytosis, hereditary elliptocytosis, spectrin, band 3

The initial fluid mosaic model for membrane structure [1] emphasized the dynamic features of membranes, with the membrane proteins viewed as capable of rapid lateral and rotational diffusion in the plane of a fluid phospholipid bilayer. The rates of diffusion of proteins were thought to be limited only by the viscosity of the local lipid environment, with no mechanism for long-range protein-protein

Received July 21, 1981; accepted August 19, 1981.

0730-2312/82/1801-0049$05.00

interactions. Many subsequent observations indicate, however, that biological membranes do not behave as simple two-dimensional solutions of proteins. Quantitative measurements of the rates of lateral diffusion of membrane proteins in a variety of systems reveal that frequently there is a population of proteins that is not mobile. Furthermore, the proteins that are mobile move at a rate 10-to 100-fold slower than predicted on the basis of membrane viscosity [2]. The rate of lateral diffusion of membrane proteins can be modulated by perturbations of the cell surface at sites separated by 10μ [3]. Furthermore, diffusion of proteins may be nonrandom in some cells [4] and can be dependent on metabolic energy as in formation of caps of surface-labelled components in lymphocytes [5]. These examples indicate possibilities for long-range interactions and organization in cell membranes and have led to proposals that at least some membrane proteins have direct interactions with underlying cytoplasmic proteins [6–8].

Association between membrane proteins and structural proteins on the inner membrane surface have been demonstrated in human erythrocytes, and the details of this linkage have been elucidated. This review will briefly cover the organization of membrane proteins in the erythrocyte, which have been discussed elsewhere [9–12], and will focus on membrane-protein associations in this cell. The erythrocyte contains a membrane-associated assembly of proteins responsible for the shape and unusual stability of this cell in the circulation. This structure is referred to as the cytoskeleton and is distinguished experimentally by the fact that it remains intact and retains the same shape as the original ghost following extraction of integral proteins and lipids with nonionic detergents [13–15]. The cytoskeleton is composed principally of spectrin, which is a phosphorylated protein, band 4.1* and erythrocyte actin as well as some other, associated polypeptides.

Spectrin has been visualized recently by low-angle rotary shadowing with platinum [16, 17] and is a flexible, rodshaped molecule about 1000 Å in length composed of two parallel polypeptide chains of 260,000 and 220,000 daltons. Spectrin heterodimers associate in solution at one end of the molecule to form tetramers, which most likely are the major form of spectrin in erythrocyte ghosts [18–20]. Purified spectrin dimer binds by lateral association with F-action [21, 22] as well as to band 4.1 [23] and also forms a complex with mixtures of these proteins [22–25]. The binding sites for band 4.1 and actin have been localized to the same region of the spectrin molecule by rotary shadowing [9, 26] suggesting the possibility of interactions between all three proteins at this site. The domain structure of spectrin has been resolved by isolation of proteolytic fragments corresponding to the sites for formation of tetramer, binding to ankyrin (see below) as well as sites of interchain association between the subunits [27, 28].

It is not clear how cross-linking occurs between spectrin, actin, and band 4.1 to form the extended anastomosing meshwork of the cytoskeleton. It is possible that erythrocyte actin, which is thought to exist as oligomers containing about ten subunits [29], may bind more than one spectrin tetramer, each of which is, in turn, linked to another actin oligomer. It has also been suggested that spectrin polypeptide chains may complex with those of adjacent tetramers to form hybrid molecules capable of multiple associations and polymerization [30].

*Nomenclature is according to Steck [11].

The erythrocyte membrane contains two major integral proteins, band 3 and glycophorin A, which span the membrane bilayer with different domains expressed on the outer and cytoplasmic surfaces [11, 12, 31]. Band 3 is a glycoprotein present in about 10^6 copies per cell and is responsible for anion transport [32]. Band 3 contains an approximately 43,000 M_r domain on the cytoplasmic surface of the membrane [33] that is associated with glycolytic enzymes aldolase and glyceraldehyde-3-phosphodehydrogenase, as well as with a 72,000 M_r peripheral membrane protein (band 4.2) of unknown function [31]. Band 3 is a stable homodimer in detergent extracts as well as in erythrocyte ghosts [34–36], and these dimers may associate to form tetramers or higher oligomers in the membrane [37, 38]. Glycophorin A (PAS 1, 2) is the major sialoglycoprotein and contains some blood group antigens and binding sites for lectins and viruses [12]. Glycophorin A is a stable dimer, even in the presence of sodium dodecyl sulphate [12], and is associated with band 3 in the membrane [39]. The association between glycophorin A and band 3 is not maintained in detergent extracts, however [34]. Band 3 and glycophorin are thought to be the major constituents of the 85 Å intramembrane particles that are visualized by freeze-fracture electron microscopy [11, 12, 31].

MEMBRANE BINDING SITE FOR SPECTRIN

Morphological experiments provided initial evidence that spectrin, and thus the cytoskeleton, is linked in some way to integral membrane proteins. Incorporation of bivalent anti-spectrin IgG into ghosts caused aggregation in the plane of the membrane of the major glycoproteins [40]. Elgsaeter and Branton subsequently reported that intramembrane particles could be aggregated by manipulations of pH and ionic strength only after removal of spectrin and actin [41]. It was also observed that spectrin could be localized on the inner surface of the membrane in patterns coincident with those of the intramembrane particles [42]. These observations led to the conclusion that the intramembrane particles and their constituent proteins were immobilized by direct association with underlying cytoskeletal proteins. This interpretation was supported by measurements of lateral mobility of band 3 and glycophorin which were restricted in the membrane, with rates of lateral displacement 10–100 times slower than expected for unrestricted movement [43–46]. An alternative explanation for restriction of the integral membrane proteins was that they simply were trapped in the underlying tangle of cytoskeletal proteins without specific protein linkages [11]. This possibility was supported by measurements of rotational diffusion of eosin-labelled band 3, which was unaffected by removal of spectrin and actin, and was consistent with unrestricted motion in the membrane [47]. Furthermore, several reports have demonstrated that spectrin can associate with artificial lipid membranes and thus may not require an integral protein to interact with the membrane [48–50].

The interaction of spectrin with erythrocyte membranes has been analysed directly by measuring reassociation of purified, radiolabelled spectrin with inside-out vesicles depleted of spectrin and actin [51–55]. Spectrin initially was metabolically labelled with $^{32}P_i$ to maintain as closely as possible the native state of the protein [51, 52], but identical measurements have also been obtained by labelling

with ^{125}I [54], reductive formylation [55], and with Bolton-Hunter reagent [56]. Spectrin has been isolated as a heterodimer in most of these studies, but quantitatively similar results have also been reported with spectrin tetramer [53]. Binding of spectrin occurred with inside-out, but not right-side-out vesicles, and exhibited an identical dependence on ionic strength, divalent metal ions, and pH as did association of spectrin with native membranes. Spectrin bound to inverted vesicles with a K_D of 10^{-8}–10^{-7} M to a single class of sites present in approximately 10^5 copies per cell. These sites involved a protein since the binding capacity of membranes was destroyed by mild proteolysis, extraction with dilute acid, or by reaction with sulfhydryl-reactive compounds such as N-ethylmaleimide. Ability of spectrin to reassociate with vesicles required an intact tertiary structure of spectrin since binding activity was destroyed by thermal denaturation in a highly cooperative manner between 49°C and 51°C. It is of interest that this is the same temperature range where erythrocytes disintegrate and where spectrin exhibits major changes in its ORD behavior [57].

The subunit of spectrin involved in membrane association has been identified as the β chain or band 2 [54, 58]. The binding site has been further localized to a 50,000 M_r fragment of the β chain produced by cleavage with 2-nitro-5-thiocyanobenzoic acid, and this binding fragment is located at the same end of the spectrin molecule involved in head-to-head association to form tetramers [27].

These experiments indicated that a specific, high-affinity protein-protein association could be reconstituted with pure spectrin and some unknown membrane component. Spectrin is confined along the membrane in erythrocyte ghosts at local concentrations that may be 10^{-5} M or higher. It thus is likely that spectrin has interactions in addition to the high-affinity site that are physiologically important but not measured by the reassociation assay. For example, spectrin may interact with membrane lipids and stabilize lipid bilayer asymmetry [50] and self-associate to form polymers [30]. The initial placement and concentration of spectrin along the membrane probably depends on the high-affinity interaction, and it has been proposed that this is an essential first event in assembly of the membrane-cytoskeleton complex [30].

IDENTIFICATION OF THE HIGH-AFFINITY SPECTRIN-BINDING PROTEIN

Initial efforts to solubilize the binding protein were not successful (V. Bennett, unpublished data) for reasons that now are known to be due to protease activity in membrane preparations. However, it was observed that controlled proteolysis of inside-out vesicles released water-soluble polypeptides that contained a competitive inhibitor of spectrin binding to membranes [59]. A 72,000 M_r polypeptide was purified from the proteolytic digest on the basis of its ability to compete for spectrin binding [59]. This polypeptide by a number of criteria had the properties expected for the membrane attachment site for spectrin. Release of the 72,000 M_r fragment from membranes paralleled closely the loss of membrane binding capacity for spectrin. The purified fragment was a potent competitive antagonist of spectrin binding, with a K_i approximately the same as the K_D for spectrin binding, to vesicles. The ability of the fragment to compete for binding was abolished by reaction with N-ethylmaleimide, which also destroyed spectrin

binding activity in vesicles. The 72,000 M_r fragment associated with spectrin in solution, and a complex of these proteins was isolated with approximately one mole of fragment per mole of spectrin heterodimer.

The 72,000 M_r fragment was released in equal amounts from vesicles prepared from untreated and α-chymotrypsin-digested erythrocytes, even though such external cleavage degrades band 3 to a 60,000 M_r fragment [59]. The 72,000 M_r fragment thus is localized exclusively on the inner surface of the membrane and cannot originate from band 3. Glycophorin and the other sialoglycoproteins could also be excluded as a source of the fragment since the periodic acid-Schiff staining profile of vesicles was unchanged by digestion, which abolished binding of spectrin and released maximal quantities of the 72,000 M_r fragment.

A 215,000 M_r polypeptide (band 2.1) localized on the inner suface of the plasma membrane has been identified as the precursor of the 72,000 M_r fragment on the basis of cross-reaction with anti-fragment antibody [60], by comparative peptide mapping [61, 62], and selective extraction of band 2.1 from membranes [60]. Antisera were raised against the fragment that had been cut from SDS-polyacrylamide gels, and monospecific anti-fragment IgG was then isolated by affinity chromatography with immobilized fragment [60]. This anti-fragment IgG was a potent inhibitor of binding of spectrin to inverted vesicles and cross-reacted only with band 2.1 and the closely migrating minor bands 2.2 and 2.3. Bands 2.2 and 2.3 are present in variable amounts in different preparation of ghosts and most likely are degradation products of band 2.1. Peptide maps were prepared from the 72,000 M_r fragment that corresponded only with band 2.1, 2.2, and 2.3 among the membrane polypeptides with a molecular weight above 72,000 M_r [61, 62]. Further evidence that the 72,000 M_r fragment is derived from band 2.1 was obtained by selective removal of band 2.1, which abolished production of the fragment and destroyed spectrin binding activity in the extracted membranes [60]. In contrast, band 4.1, which has been identified as a spectrin-binding protein in the cytoskeleton [24–26], was extracted completely with no effect on binding of spectrin or production of the 72,000 M_r fragment [60].

Direct evidence that spectrin associated with band 2.1 in membranes was provided by isolation with anti-fragment IgG of a specific complex of spectrin and 2.1 in a 1:1 molar ratio formed during reassociation of spectrin with inverted vesicles [60]. Furthermore, partially purified 2.1 was a competitive antagonist of spectrin binding to inverted vesicles. It was concluded from these experiments and from the evidence that 2.1 was the precursor of the 72,000 M_r fragment that 2.1 was the high-affinity membrane attachment site for spectrin. In recognition of this binding function, the protein was named ankyrin, from the Greek "ankyra," which means anchor [60].

Ankyrin has been purified in milligram amounts as a water-soluble protein in the absence of detergent [23, 26, 63]. The isolated protein contains a single polypeptide chain and is somewhat asymmetric with a frictional ratio of 1.46 [63]. Ankyrin has some hydrophobic character based on the behavior in charge-shift electrophoresis but is much less hydrophobic than an integral membrane protein such as band 3 [63]. Ankyrin as isolated from the membrane is the same protein identified as the spectrin-binding site since this protein can be almost completely immunoprecipitated by anti-72,000 M_r fragment IgG [63]. Furthermore, controlled digestion of purified ankyrin produces a 72,000 M_r

fragment as well as fragments corresponding to bands 2.2 and 2.3 [63]. Ankyrin binds to spectrin in solution with high affinity (K_D 5 × 10^{-8} M) at the same site as the 72,000 M_r fragment and forms a complex with a stoichiometry of one mole of ankyrin per mole of spectrin heterodimer [26, 63]. Association of ankyrin with spectrin has been visualized by low-angle rotary shadowing [23, 26] and occurs at a site on spectrin about 200 Å from the head of the molecule where spectrin heterodimers join to form a tetramer.

Ankyrin is present in erythrocytes in about 100,000 copies per cell, as determined by radioimmunoassay with anti-fragment IgG [64]. Spectrin heterodimers, on the other hand, are present in about 200,000 copies per cell [11]. It is likely that the spectrin molecules present in excess over ankyrin binding sites are associated with membrane-bound spectrin to form tetramers. Two lines of evidence support this conclusion. Spectrin most likely is a tetramer or higher-order oligomer in erythrocyte membranes based on chemical cross-linking experiments [20], presence of spectrin tetramer in membrane extracts prepared under mild conditions [18], and characterization of the dimer-tetramer equilibrium [24]. Furthermore, spectrin tetramer associates with vesicles with the same affinity and with the same number of binding sites as spectrin heterodimers [53]. It thus seems likely that association of spectrin with the membrane occurs independently of the self-association to form tetramers and that a spectrin tetramer binds to the membrane at only one of its two potential ankyrin sites. The reason that spectrin tetramers bind monovalently may be that the distance between ankyrin sites on the membrane are greater than the distance between binding sites on the tetramers or that occupation of one ankyrin site in some way inhibits binding at the second site.

ASSOCIATION OF ANKYRIN AND BAND 3

The fact that ankyrin is a water-soluble protein raises new questions about the linkage between spectrin and integral membrane proteins. If it is correct that spectrin is associated with intramembrane particles [40, 42] and that ankyrin is the binding protein, then ankyrin should provide a linkage between spectrin and some component of the complex of proteins that form the intramembrane particles. Several different types of experiments have demonstrated that, in fact, ankyrin is directly associated with band 3 at its 43,000 M_r cytoplasmic domain. Anti-ankyrin IgG co-precipitates band 3 with ankyrin in a 1:1 molar ratio from detergent extracts of spectrin-depleted vesicles [65]. The association of band 3 with ankyrin was specific since the sialoglycoproteins (glycophorins) were not present and since band 3 was not immunoprecipitated following denaturation of the extract [65]. The ankyrin-associated band 3 was nearly identical to the free population of band 3 on the basis of its CNBr fragments and also was demonstrated to span the membrane [65]. Furthermore, antibodies raised against the 43,000 M_r cytoplasmic domain of band 3 cross-reacted with the ankyrin-linked band 3 [66].

Some band 3 remains firmly associated with cytoskeletal proteins and persists after repeated washes in detergent [65, 67]. This fraction of band 3, which represents about 10^5 copies per cell, is most likely associated with ankyrin, which in turn is bound to spectrin. This conclusion is based on the findings that spectrin binds to ankyrin-linked band 3 but not free band 3 [65] and that band 3 will rebind to cytoskeletal assemblies that contain ankyrin but not to ankyrin-depleted cytoskeletons (V. Bennett, unpublished data).

The fact that band 3 and ankyrin were immunoprecipitated from detergent extracts by anti-ankyrin IgG in a 1:1 molar ratio and that band 3 is a stable dimer in detergent [34] led to the suggestion that two ankyrin molecules were associated with a band 3 dimer [65]. This is not the case, however, since the ankyrin band 3 oligomer has been purified and characterized (V. Bennett, manuscript in preparation) and contains one molecule of ankyrin and one molecule of band 3. Ankyrin thus is most likely distributed randomly among band 3 dimers.

The cytoplasmic domain of band 3 is released by mild proteolysis and has been purified [66]. Ankyrin binds to this band 3 fragment in solution with a K_D of 5×10^{-9} M and in a 1:1 molar ratio [66]. This observation demonstrates that ankyrin can bind directly to a specific domain of band 3 without assistance of intermediary proteins. Ankyrin radiolabelled with ^{125}I-Bolton-Hunter reagent will reassociate with inverted vesicles that have been depleted of ankyrin as well as most of the peripheral membrane proteins [66, 68]. Band 3 is the binding site for ankyrin since the reassociation is abolished by anti-band 3 fragment IgG, by selective proteolytic cleavage of band 3, and by the cytoplasmic fragment of band 3. Furthermore, ankyrin binds to vesicles and to liposomes reconstituted with band 3 in a nearly identical manner.

Measurements of ankyrin reassociation with membranes and liposomes have demonstrated convincingly that ankyrin is binding directly to band 3. However, these studies have also raised some new questions that remain unanswered. The maximal number of binding sites for ankyrin is at most 200,000 copies per cell [56, 66, 68], while band 3 is present in about 10^6 copies per cell. It is unlikely that ankyrin is binding to a subpopulation of band 3 since the free population of band 3 isolated by extraction of cytoskeletons that retain ankyrin-linked band 3 can still bind to ankyrin when reconstituted into liposomes [68] or in solution [63]. Furthermore, ankyrin-linked band 3 has been purified as an oligomeric complex with ankyrin and was found to be nearly identical to the free population of band 3 by two-dimensional peptide mapping (V. Bennett, manuscript in preparation). Another possibility consistent with available data is that ankyrin binds to band 3 only in certain oligomeric states, such as a tetramer but not a monomer or dimer. It is pertinent in this regard that band 3 may exist as a tetramer in erythrocyte ghosts [37, 38] and, in this case, would be present in near equivalence to the number of ankyrin sites as about 250,000 assemblies per cell.

A further complexity of ankyrin rebinding is that the 43,000 M_r fragment of band 3 competes for ankyrin binding with a much lower affinity than this fragment binds to ankyrin in solution [66, 68]. Moreover, Scatchard plots of binding data are curvilinear [56, 66] and thus consistent with either two distinct types of sites, one site that can have either high or low affinity depending on unknown factors or with a negatively cooperative type of interaction of ankyrin with a single class of sites. The conclusion from these experiments is that details of ankyrin-band 3 association in membranes are not understood and that this linkage is more complex than the ankyrin-spectrin interaction.

IMPLICATIONS FOR THE ERYTHROCYTE MEMBRANE

Ankyrin thus performs dual functions in the erythrocyte membrane of (a) providing the high-affinity attachment of spectrin to the cytoplasmic surface and thus allowing assembly of the cytoskeleton and (b) linking the membrane bilayer

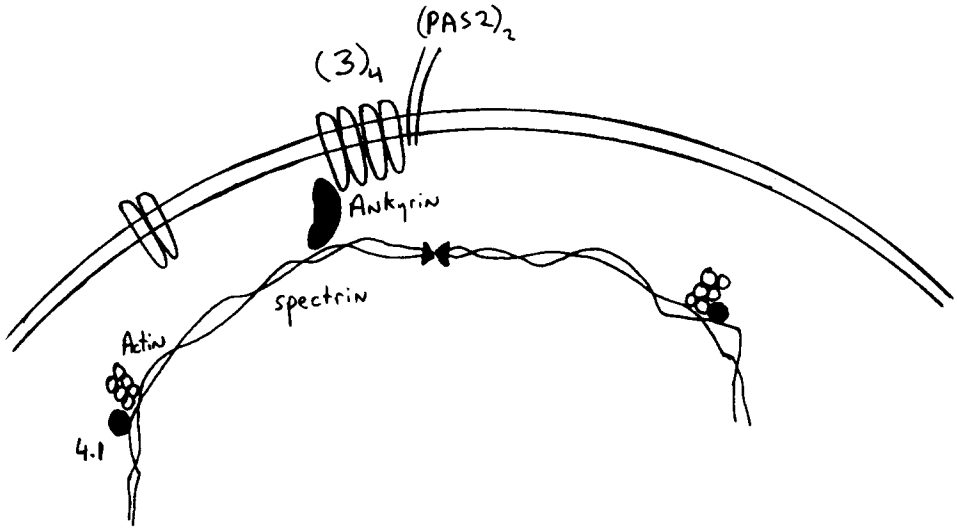

Fig. 1. Model for organization of the major structural proteins in the human erythrocyte membrane. Nomenclature is according to Steck [11].

through integral membrane proteins to the underlying cytoskeleton (Fig. 1). It is evident, intuitively, that linkage between the fragile bilayer and the cytoskeleton is essential for maintaining a stable cell shape. Ankyrin is present in about 10^5 copies per cell and is thus associated with 10^5 band 3 molecules that are components of intramembrane particles. The ankyrin-particle stoichiometry is not known but would be 1:1 if ankyrin is distributed randomly, corresponding to 10^5 cytoskeleton-linked particles. The average nearest neighbor distance between ankyrin-linked particles would be about 350 Å, which is comparable to the apparent size of spectrin tetramer in solution [69] and considerable less than the dimensions of rotary-shadowed spectrin [16, 17]. Linkage of the membrane to the spectrin assembly at these intervals would only allow evagination of vesicles with diameters 600 Å or less, which approaches the minimal limit for membranes observed with artificial bilayer vesicles. During maturation of erythrocytes, membrane vesicles are released from spectrin-free areas [70], and this process presumably continues until the distance between membrane anchoring sites decreases to a point where no further vesiculation occurs.

The fact that only a fraction of band 3 molecules is linked to the cytoskeleton is in agreement with measurements of the rotational diffusion of band 3, which indicated an unhindered local environment and little interaction with spectrin [47]. More detailed measurements of band 3 rotational diffusion have resolved a population of molecules with decreased mobility that can be freed from restraints by cleavage of the cytoplasmic domain or by extraction of ankyrin and band 4.2 with high salt [71]. About 40% of band 3 is immobilized in this way, which is consistent with ankyrin linkage to 10^5 band 3 dimers that are in equilibrium with another 10^5 dimers to form band 3 tetramers [37, 28].

Association of spectrin with integral proteins has a substantial effect on their lateral mobility in the plane of the membrane. Band 3 exhibited a 50-fold higher rate of diffusion in erythrocyte membranes from a strain of mice deficient in cytoskeletal proteins including spectrin [45]. Another approach to study effects of cytoskeletal associations has been to expose erythrocyte ghosts to conditions of low ionic strength and elevated temperature that promoted dissociation of spectrin from its membrane site and increased the diffusion constant for band 3 up to 50-fold [46]. The 72,000 M_r spectrin-binding fragment of ankyrin has been used to dissociate selectively spectrin from the membrane, and this treatment increased the rate of diffusion of fluorescein isothiocyanate-labelled integral proteins in Sendai virus-fused erythrocytes [72].

Only a fraction of band 3 proteins interact with high affinity with spectrin, and yet over 90% of these proteins are restricted in lateral movement [46]. It is possible that immobilization results from lower-affinity interaction of band 3 with spectrin or other cytoskeletal proteins. Another possibility that may occur in parallel is that band 3 tetramers associate with each other with low affinity, which would decrease the diffusion constant due to increased effective size of the aggregate, and would immobilize those proteins associated with an ankyrin-linked tetramer. These alternatives could be resolved by measurements of diffusion as a function of integral protein density, since interaction with cytoskeletal elements would be independent of this parameter, and protein-protein interactions would be highly sensitive.

Regulation of membrane properties by modulation of protein-protein associations is an intriguing possibility, especially in view of the fact that spectrin [73, 74], ankyrin [60] and the cytoplasmic domain of band 3 [60] are all phosphorylated in whole cells. Furthermore, crenation of cells accompanies dephosphorylation of spectrin during metabolic depletion of ATP, and conversely, ATP-dependent phosphorylation of spectrin has been correlated with restoration of normal disc morphology [75, 76]. However, phosphorylation of spectrin has no effect on association with ankyrin since cleavage of phosphate groups with phosphatase or proteolytic removal of the phosphorylated peptides has no effect on spectrin binding to membranes [77]. Furthermore, dephosphorylation of ankyrin and band 3 by phosphatase has no effect on ankyrin-spectrin or ankyrin-band 3 association (V. Bennett, unpublished data). The phosphorylated state of spectrin also has no influence on binding to F-actin [21], or on self-association to form tetramers [24]. The function of phosphorylation in the erythrocyte membrane thus remains obscure. It will be of interest to examine the effect of carboxylmethylation in this system since modification of band 3 by methylation has been reported in ghosts [78].

Band 3 contains the anion transport channel [32] as well as binding sites for band 4.2 and glycolytic enzymes [31]. Ankyrin association with membranes is unaltered by aldolase and glyceraldehyde-3-phosphodehydrogenase (V. Bennett, unpublished data), and thus binds to a different site on band 3. The effect of other band 3 functions on linkage to the cytoskeleton and vice versa remain to be studied. Ankyrin-linked band 3 contains a DIDS reactive site (V. Bennett, manuscript in preparation), and thus presumably has the potential for anion transport [32], although this has not been demonstrated directly by transport measurements.

MEMBRANE-CYTOSKELETON ASSOCIATION IN ABNORMAL ERYTHROCYTES

A sequence of protein-protein linkages have been outlined that connect the cytoskeleton to the membrane and that presumably are essential for normal membrane function. It is thus reasonable to predict that fragile or abnormally shaped erythrocytes from at least some patients with hereditary hemolytic anemias would have a defect in one or more of these proteins. Several examples of such defects have, in fact, been detected. Pyropoikilocytosis is a rare disorder in which erythrocytes fragment and assume bizarre shapes after warming to 45–46°C, which is about 4°C less than the disintegration temperature for normal erythrocytes [79]. Spectrin isolated from these cells denatures at the same temperature of 45–46°C based on change in the ORD spectrum, while normal spectrin denatures at 49°C. It is not known which functions of pyropoikilocytic spectrin are lost after heating, but this may include ability to bind to the membrane. A shift of about one degree in the thermal denaturation curve for spectrin has also been reported in some cases of hereditary nonhemolytic elliptocytosis, although not all patients with this clinical presentation have such as shift [80].

A 50% loss of high-affinity binding sites for ankyrin have been measured in erythrocyte membranes in two families with a hemolytic anemia [56]. Erythrocytes from splenectomized patients in these families were microcytic and exhibited bizarre, irregular shapes. The defective protein in these cells has not been determined, although it is known that the cytoplasmic domain of band 3 purified from the defective cells binds to ankyrin in a normal manner [56]. These erythrocytes may have an altered arrangement of ankyrin binding sites rather than a defect in the binding site itself. In any event, this example demonstrates the importance of the cytoskeleton-membrane linkage in maintaining normal cell morphology.

Spectrin binding sites and ability of spectrin to bind to normal membranes was unaltered in the erythrocytes deficient in high-affinity ankyrin binding and also was normal in several cases of hereditary spherocytosis [56]. These results do not rule out defects in spectrin-membrane association in all forms of spherocytosis, however, since this disease is described on the basis of morphology and could result from more than one type of biochemical defect. Further study of protein associations in abnormal cells promises to provide clinical correlates for the biochemical studies and to offer the equivalent of genetics for probing in more detail the structure of normal membranes.

ERYTHROCYTE PROTEIN ANALOGUES IN OTHER CELL TYPES

The erythrocyte cytoskeleton is a remarkable, highly specialized structure that stabilizes the membrane of otherwise fragile cells. Several proteins of the erythrocyte system have analogues in other more complex cell types. Spectrin, filamin, and actin binding protein from macrophages have a similar and distinctive extended rod-like appearance in rotary-shadowed replicas [81]. Furthermore, all three proteins form higher oligomers by head-to-head association. Filamin lacks the membrane binding activity of spectrin [81; V. Bennett, unpublished data] and thus is not analogous to the β subunit of spectrin that contains the ankyrin binding site [54, 58]. Filamin and macrophage actin-binding protein comigrate

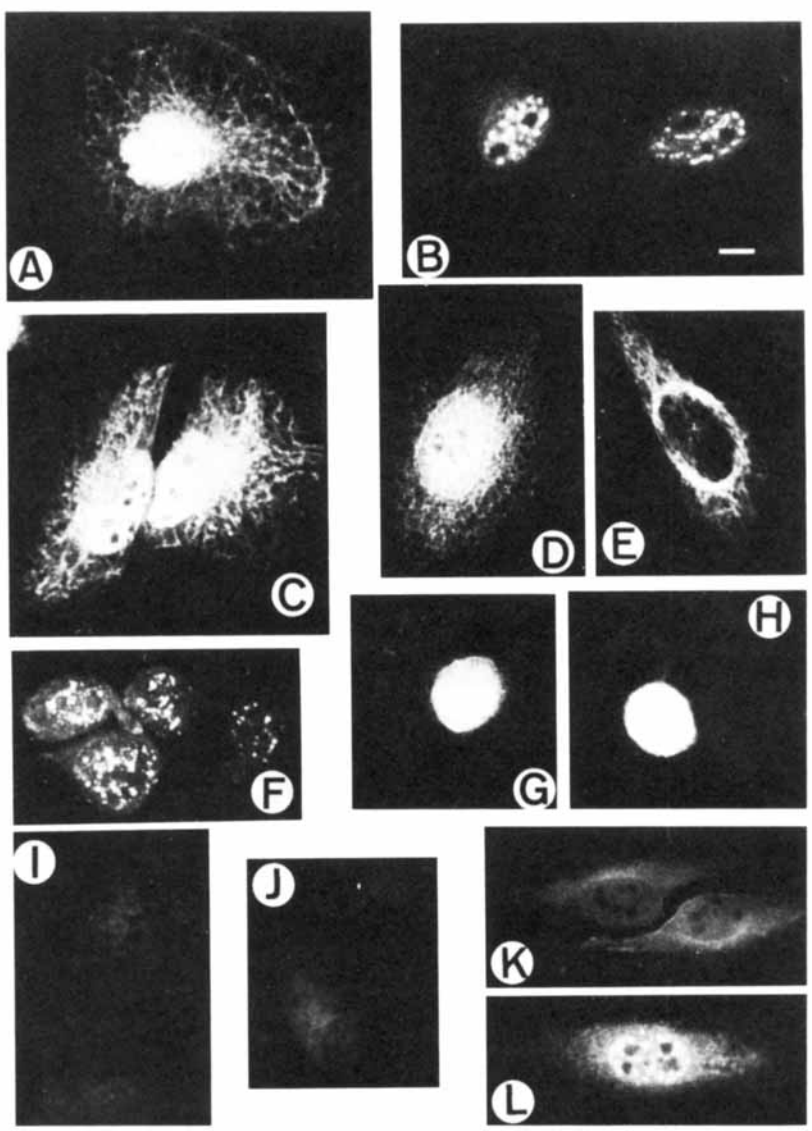

Fig. 2. Indirect immunofluorescent staining of HeLa cells with monospecific anti-ankyrin IgG. HeLa cells on coverslips were fixed with formaldehyde, permeabilized with Triton X-100 and incubated with 16 nM anti-ankyrin IgG or 20 nM preimmune IgG (J), followed by rhodamine-labelled goat anti-rabbit IgG. The negative in panel B was over-exposed to reveal details of the nuclear staining. In panel F, cells were extracted with Triton X-100 before fixation with formaldehyde. Cells in panels G and H were chilled to 4°C for 60 min and then incubated 120 min at 37°C with 10 μM colchicine, while cells in panel D were chilled and warmed at the same time in the absence of colchicine. Controls (I–L) were with 50 nM ankyrin and anti-ankyrin IgG (I), preimmune IgG (J), anti-ankyrin and 100 μg/ml brain MAP fraction (K), and anti-ankyrin and 100 μg/ml brain tubulin (L). The bar represents 10 μ [82].

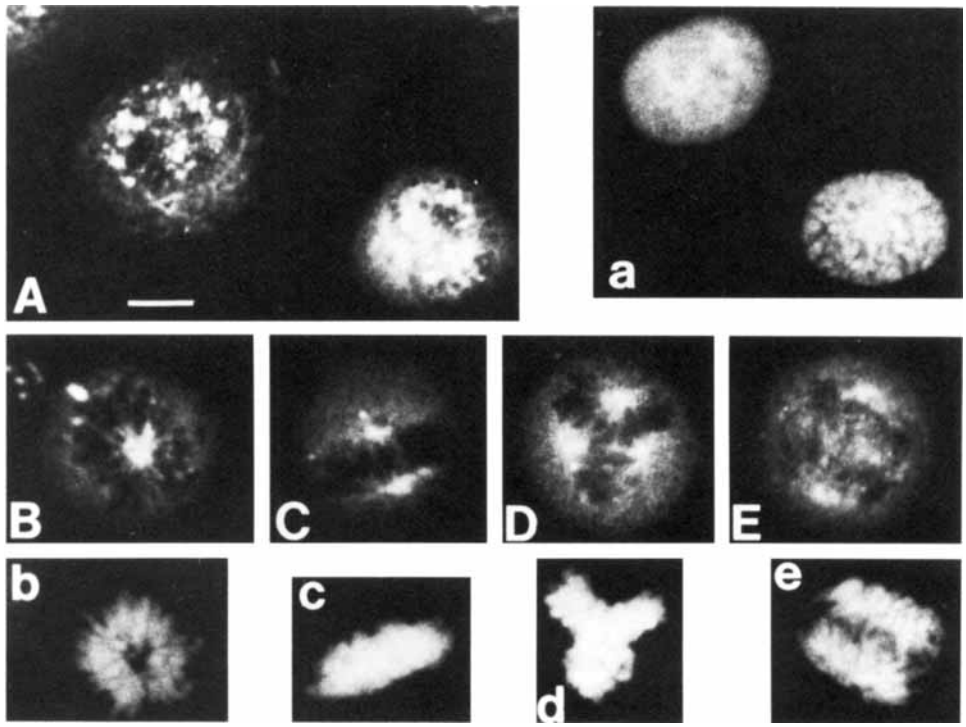

Fig. 3. Double labeling of formaldehyde-fixed Chang liver cells undergoing mitosis with a DNA-specific dye and by indirect immunofluroescence with monospecific anti-ankyrin IgG. Chang liver cells were grown and stained with anti-ankyrin IgG (Fig. 2), followed by a 15-min incubation at 0°C with 2.5 μg/ml bisbenzimide (Hoechst 33258) in extraction buffer. The coverslips were then washed three times and processed as usual. Panels A–E are the rhodamine fluorescence, while a–e are the bisbenzimide fluorescence. The bar represents 10μ [82].

with the α subunit of spectrin of SDS gels, and the α subunit also contains the domain responsible for head-to-head association of spectrin dimers [27, 28]. It thus seems that the α subunit of spectrin is closely related to the high molecular weight actin-binding proteins, while analogues for the membrane-attachment β subunit remain to be identified. It is possible that such a membrane-binding subunit is present in cells but is dissociated from the actin-binding subunit during the purification procedure.

Immunoreactive forms of ankyrin have been detected by radioimmunoassay in platelets, neutrophils, and platelets in about the same amount as in erythrocytes when normalized by surface area [64]. Immunoreactivity also was measured in cultured human fibroblasts and in rat brain, liver, kidney, testis, and adipocytes [64]. A protein of about 370,000 M_r comigrating with MAPI has been identified in soluble brain extracts that cross-reacts with ankyrin and is a microtubule-associated protein [82]. In addition, a major portion of ankyrin immunoreactivity in brain extracts is present as a pair of polypeptides of approximately 200,000 and 220,000 M_r that do not associate with microtubules and may be proteolytic fragments of the 370,000 M_r polypeptide. In HeLa cells,

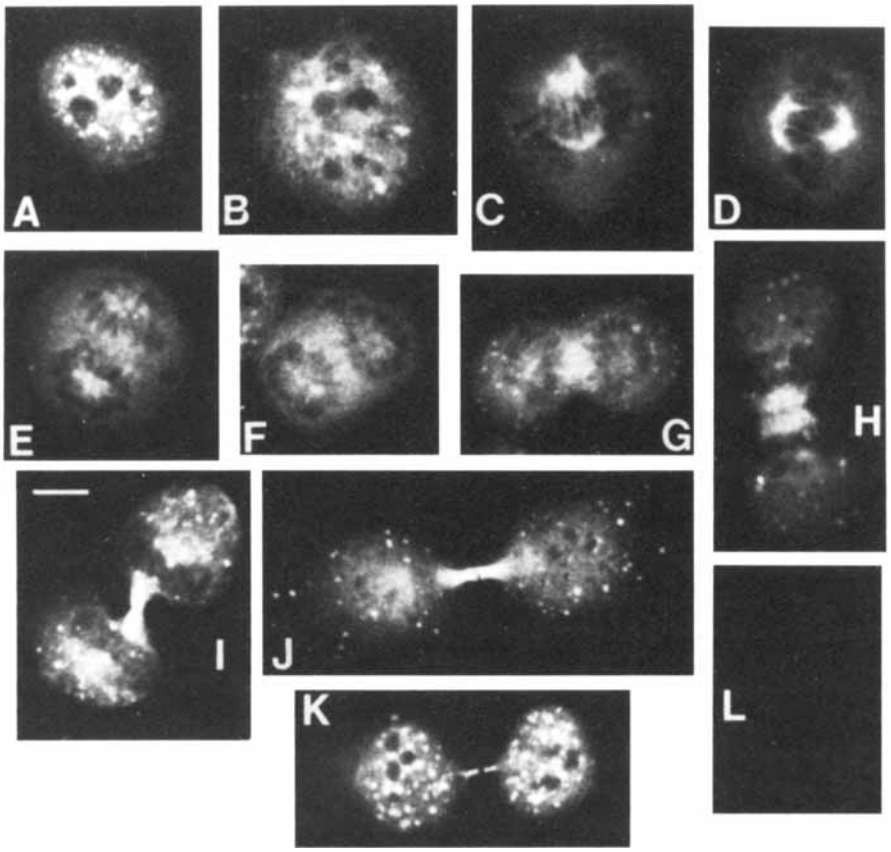

Fig. 4. Indirect immunofluorescent staining of formaldehyde-fixed Chang liver cells during various stages of mitosis with monospecific anti-ankyrin IgG. The panels are arranged in a probable sequence of mitosis starting with interphase (A), prophase (B), metaphase (C,D), late anaphase (E,F), and telophase (G–J) and early interphase with retention of the midbody (K). Panel L was a control sample which contained ankyrin (250 nM) during incubation with the first antibody. The bar represents 10 μ [82].

the major cross-reacting proteins are polypeptides of 220,000 and 35,000 M_r, and a minor band of 250,000 M_r that may be the precursor of these polypeptides.

The distribution of ankyrin immunoreactivity has been visualized by indirect immunofluorescence in cultured cells (Figs. 2–4) [82] and is localized in a filamentous meshwork surrounding the nucleus and extending throughout the cytoplasm and in an intensely staining punctate pattern over nuclei. The cytoplasmic meshwork resembles closely patterns obtained with anti-tubulin fluorescence [83, 84]. A possible microtubule-association of ankyrin fluorescence is also suggested by the sensitivity to colchicine which abolished the cytoplasmic staining (Fig. 2). The punctate nuclear staining has not been observed with tubulin but is related to microtubule-associated proteins since this is displaced entirely by partially purified MAP1 from brain (Fig. 2). Pure tubulin, in contrast, had no effect.

The distribution of ankyrin fluorescence changes dramatically during mitosis (Figs. 3, 4) [82] with rearrangements that parallel closely the behavior of tubulin [83, 84] and microtubule-associated proteins [85–87]. In early prophase, the cytoplasmic meshwork is replaced by a diffuse staining throughout the cytoplasm, while in metaphase cells stain is concentrated above and below the assembled chromosomes at the spindle poles. By late anaphase, fluorescence still is present at the poles, but some also appears at the midregion of the cell where cytokinesis will occur. Constriction of the cell, which marks onset of cytokinesis, is accompanied by intense staining of the midregion of the cell where the cleavage furrow is located and loss of stain at the spindle poles. Towards the end of telophase, daughter cells have formed but remain connected by a narrow intercellular bridge, which is intensely fluorescent except at the midbody where no staining occurs.

The findings that ankyrin analogues are localized in a microtubule-like pattern in interphase and dividing cells, and are associated with microtubules in brain suggest that ankyrin in erythrocytes has evolved from a microtubule-associated protein. Mature human erythrocytes lack tubulin, but tubulin is present in a membrane-associated marginal band in mammalian erythroblasts as well as primitive circulating fetal erythrocytes [88]. Furthermore, a protein similar to a high molecular weight microtubule-associated protein from brain has been identified in the marginal band of nucleated erythrocytes [89]. Direct evidence for a tubulin-related activity for ankyrin is that ankyrin binds to pure brain tubulin in a saturable manner manner and at the same site as a brain microtubule-associated protein [82].

The extent of homology between ankyrin and its analogues remains to be established by peptide mapping and amino acid sequence analysis of the purified proteins. It is likely, however, that ankyrin analogues will not be as closely conserved as the different forms of actin or calmodulin but will represent a related family of proteins such as the different intermediate filament proteins [90] and myosins [91, 92].

Ankyrin performs in erythrocytes as a linkage protein, and its analogues may function similarly as bridge proteins. For example, microtubules interact with membranes by an unknown mechanism [93–95] and are involved in maintaining the Golgi apparatus in a juxtanuclear position [96]. Microtubules also are associated morphologically with intermediate filaments [97] and interact with actin [98]. Ankyrin analogues, perhaps in concert with spectrin-like proteins, may act as the linkage protein in some of these microtubule associations. The localization of ankyrin staining over spindle poles during metaphase and anaphase parallels closely the arrangement of membranes of the nuclear envelope and endoplasmic reticulum in dividing cells [99, 100]. The spindle-associated membrane system is thought to regulate calcium levels and to perform an anchorage function in stabilization of the spindle apparatus. A membrane linkage also is required in the cleavage furrow where ankyrin staining is intense. Ankyrin-like polypeptides are a logical candidate for the bridge protein in these membrane associations.

CONCLUSION

The studies discussed in this review have led to a detailed understanding of membrane-protein interactions in the human erythrocyte, which promises to be of clinical significance and to have direct relevance to other cell types. The strategy

of these studies of sequential removal of one protein followed by its purification, reassociation, and identification of its binding site also may have application in elucidating details of other complex membrane structures. For example, desmosomes, postsynaptic densities, Z-discs, and adhesion plaques most likely represent stable assemblies of protein components that are ultimately attached to the membrane and would be amenable to such an approach. It is probable, based on the erythrocyte analogy, that end-on attachment of filament-forming proteins such as actin or intermediate filament proteins to membranes will require several intermediate proteins that may themselves be associated to form an extensive, lateral structure.

ACKNOWLEDGMENTS

Research by the author was carried out in the laboratory of D. Branton, Harvard University from 1976 to 1977, at the Wellcome Research Laboratories from 1977 to 1980 and has been subsequently funded by grants from the National Institutes of Health (RCDA and research grant 1RO1 AM29808-1), Muscular Dystrophy Association, and the Burroughs Wellcome Fund. Valuable assistance by P. Stenbuck and J. Davis is gratefully acknowledged.

REFERENCES

1. Singer SJ, Nicolson GL: Science 175:720, 1972.
2. Cherry RJ: Biochim Biophys Acta 559:289, 1979.
3. Schlessinger J, Elson EL, Webb WW, Yahara I, Rutishauser U, Edelman GM: Proc Natl Acad Sci USA 74:1110, 1977.
4. Smith BA, Clark WR, McConnell HM: Proc Natl Acad Sci USA 76:5641, 1979.
5. Unanue ER, Karnovsky MJ: Transplant Rev 14:184, 1973.
6. Edelman GM: Science 192:218, 1976.
7. Nicolson GL: Biochim Biophys Acta 457:57, 1974.
8. Singer SJ: Annu Rev Biochem 43:805, 1974.
9. Branton D, Cohen CM, Tyler J: Cell 24:24, 1981.
10. Lux SE: Semin Hematol 16:21, 1979.
11. Steck TL: J Cell Biol 62:1,1974.
12. Marchesi VT, Furthmayr H, Tomita M: Annu Rev Biochem 45:667, 1976.
13. Yu J, Fischman DA, Steck TL: J Supramol Struct 1:233, 1973.
14. Lux SE, John KM, Karnovsky MJ: J Clin Invest 58:955, 1976.
15. Sheetz MP, Sawyer D: J Supramol Struct 8:399, 1978.
16. Shotton DM, Burke BE, Branton D: Biochim Biophys Acta 536:313, 1978.
17. Shotton DM, Burke BE, Branton D: J Mol Biol 131:303, 1979.
18. Ralston GB: Biochim Biophys Acta 455:163, 1976.
19. Ungewickell E, Gratzer W: Eur J Biochem 88:379, 1978.
20. Ji TH, Kiehm DJ, Middaugh GR: J Biol Chem 255:2990. 1980.
21. Brenner SL, Korn ED: J Biol Chem 254:8620, 1979.
22. Cohen CM, Tyler JM, Branton D: Cell 21:875, 1980.
23. Tyler JM, Hargreaves WR, Branton D: Proc Natl Acad Sci USA 76:5192, 1979.
24. Ungewickell E, Bennett PM, Calvert R, Ohanian V, Gratzer W: Nature (London) 280:811, 1979.
25. Fowler V, Taylor DL: J Cell Biol 85:361, 1980.
26. Tyler JM, Reinhardt GN, Branton D: J Biol Chem 255:7034, 1980.
27. Morrow J.S. Speicher DW, Knowles WJ, Hsu CJ, Marchesi VT: Proc Natl Acad Sci USA 77:6592, 1980.
28. Speicher DW, Morrow JS, Knowles WJ, Marchesi VT: Proc Natl Acad Sci USA 77:5673, 1980.
29. Brenner SL, Korn ED: J Biol Chem 255:1670, 1980.

30. Morrow JS, Marchesi VT: J Cell Biol 88:463, 1981.
31. Steck TL: J Supramol Struct 8:311, 1978.
32. Cabantichik ZI, Knauf PA, Rothstein A: Biochim Biophys Acta 515:239, 1978.
33. Steck TL, Ramos B, Strapazon E: Biochemistry 15:1154, 1976.
34. Yu J, Steck TL: J Biol Chem 250:9176, 1975.
35. Steck TL: J Mol Biol 66:295, 1972.
36. Nigg E, Cherry RJ: Nature (London) 277:493, 1979.
37. Weinstein RS, Khodadad JK, Steck TL: In Lassen UV, Ussing HH, Wieth JO (eds): "Membrane Transport in Erythrocytes." Copenhagen: Manksgaard, 1980, p 35.
38. Nigg EA, Cherry RJ: Biochemistry 18:3457, 1979.
39. Nigg EA, Bron C, Girardet M, Cherry RJ: Biochemistry 19:1887, 1980.
40. Nicolson GL. Painter RG: J Cell Biol 59:395, 1973.
41. Elgsaeter A, Branton D: J Cell Biol 63:1018, 1974.
42. Shotton DM, Thompson K, Wofsy L, Branton D: J Cell Biol 76:512, 1978.
43. Peters R, Peters J, Tews KH, Bahr W: Biochim Biophys Acta 367:282, 1974.
44. Fowler V, Branton D: Nature (London) 268:23, 1977.
45. Sheetz MP, Schindler M, Koppel D: Nature (London) 285:510, 1980.
46. Golan DE, Veatch W: Proc Natl Acad Sci USA 77:2537, 1980.
47. Cherry RJ, Burkli A, Busslinger M, Schneider G, Parish GR: Nature (London) 263:389, 1976.
48. Mombers C, Van Dijck PWM, Van Deenen LLM, De Gier J, Verkleij AJ: Biochim Biophys Acta 470:152, 1977.
49. Mombers C, Verkleij AJ, De Gier J, Van Deenen LLM: Biochim Biophys Acta 551:271, 1979.
50. Haest CWM, Plasa G, Kamp D, Deuticke B: Biochim Biophys Acta 509:21, 1978.
51. Bennett V, Branton D: J Biol Chem 252:2753, 1977.
52. Bennett V: Life Sci 21:433, 1977.
53. Goodman SR, Weidner SA: J Biol Chem 255:8082, 1980.
54. Litman D, Hsu CJ, Marchesi VT: J Cell Sci 42:1, 1980.
55. Baskin GS, Langdon RG: J Biol Chem 256:5428, 1981.
56. Agre P, Orringer E, Chui D, Bennett V: J Clin Invest (in press).
57. Brandts JF, Erickson L, Lysko K, Schwartz AT, Taverna RD: Biochemistry 16: 3450, 1977.
58. Calvert R, Bennett P, Gratzer W: Eur J Biochm 107:355. 1980.
59. Bennett V: J Biol Chem 253:2292, 1978.
60. Bennett V, Stenbuck PJ: J Biol Chem 254:2533, 1979.
61. Luna EJ, Kidd GH, Branton D: J Biol Chem 254:2526, 1979.
62. Yu J, Goodman SR: Proc Natl Acad Sci USA 76:2340, 1979.
63. Bennett V, Stenbuck PJ: J Biol Chem 255:2540, 1980.
64. Bennett V: Nature (London) 281:597, 1979.
65. Bennett V, Stenbuck PJ: Nature (London) 280:468, 1979.
66. Bennett V, Stenbuck PJ: J Biol Chem 255:6424, 1980.
67. Sheetz MP: Biochim Biophys Acta 557:122, 1979.
68. Hargreaves WR, Giedd KN, Verkleij A, Branton D: J Biol Chem 255:11965, 1980.
69. Ralston GB: J Supramol Struct 8:361, 1978.
70. Tokuyasu KT, Schekman R, Singer SJ: J Cell Biol 80:481, 1979.
71. Nigg EA, Cherry RJ: Proc Natl Aca Sci USA 77:4702, 1980.
72. Fowler V, Bennett V: J Supramol Struct 8:215, 1978.
73. Palmer FB, Verpoorte J: Can J Biochem 49:337, 1971.
74. Harris HW, Lux SE: J Biol Chem 255:11512, 1980.
75. Sheetz MP, Singer SJ: J Cell Biol 73:638, 1977.
76. Birchmeier W, Singer SJ: J Cell Biol 73:647, 1977.
77. Anderson JM, Tyler JM: J Biol Chem 255:1259, 1980.
78. Terwilliger TC, Clarke S: J Biol Chem 256:3067, 1981.
79. Chang K, Williamson JR, Zarkowsky HS: J Clin Invest 64:326, 1979.
80. Tomaselli MB, John KM, Lux SE: Proc Natl Acad Sci USA 78:1911, 1981.
81. Tyler JM, Anderson JM, Branton D: J Cell Biol 85:489, 1980.
82. Bennett V, Davis J: Proc Natl Acad Sci USA (in press).
83. Brinkley BR, Fuller G, Highfield D: In Goldman et al (eds): "Cell Motility." Coldspring Harbor Conferences on Cell Proliferation, 1976, p 435.

84. Weber K: In Goldman et al (eds): "Cell Motility." Coldspring Harbor Conferences on Cell Proliferation, 1976, p 403.
85. Connolly JA, Kalnins VI, Cleveland DW, Kirschner MW: J Cell Biol 76:781, 1978.
86. Sherline P, Schiavone K: Science 198:1038, 1977.
87. Connolly JA, Kalnins V, Cleveland D, Kirschner MW: Proc Natl Acad Sci USA 74:2437, 1977.
88. Van Deurs B, Behnke O: Z Anatentwicklungsgesch 143:43, 1973.
89. Sloboda RD, Dickersin K: J Cell Biol 87:170, 1980.
90. Lazarides E: Nature (London) 283:249, 1980.
91. Pollard TD, Stafford W, Porter M: J Biol Chem 253:4798, 1978.
92. Gadasi H, Korn ED: Nature (London) 286:452, 1980.
93. Bhattacharyya B, Wolff J: J Biol Chem 250:7639, 1975.
94. Stephens RE: Biochemistry 16:2047, 1977.
95. Sherline P, Lee YC, Jacobs L: J Cell Biol 72:380, 1977.
96. Moskalewski S, Thyberg J, Hinek A, Friberg A: Tissue and Cell 9:185, 1977.
97. Geiger B, Singer SJ: Proc Natl Acad Sci USA 77:4769, 1980.
98. Griffith L, Pollard TD: J Cell Biol 78:958, 1978.
99. Harris P: Exp Cell Res 94:409, 1975.
100. Hepler P: J Cell Biol 86:490, 1980.

NOTE ADDED IN PROOF

An additional membrane-cytoskeletal linkage has been suggested, involving association between band 4.1 and a minor sialoglyco-protein (glycophorinc) (Mueller T, Morrison M: In Kruckberg et al (eds): Erythrocyte Membranes 2: Recent Clincal and Experimental Advances, pp 95–112, Alan R Liss Inc, New York, 1981).