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Substructure of Human Erythrocyte Spectrin

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The human erythrocyte structural protein spectrin and its subunits I, II were isolated in the presence of Na-dodecyl-sulfate by gel filtration and preparative gel electrophoresis. After removal of the detergent, spectrin alpha-helical content is comparable to spectrin isolated without detergent. Subunits I and II formed single bands in isoelectric focusing (pI = 5.6) and in Ornstein-Davis disc gel electrophoresis systems, indicating the individual subunits are homogenous in nature. The molecular weights of the subunits I and II, determined by Ferguson plot, are 237,500 and 238,600, respectively, which is in good agreement with values obtained by the standard SDS gel relative mobility method. Limited tryptic digestion of spectrin and two-dimensional peptide maps of the individual subunits cleaved by S-cyanylation reaction showed dissimilar patterns, suggesting differences in primary structure between the two subunits.

Key words: spectrin, actin, red cell membranes

Many current models of membrane organization favor the idea that transmembrane glycoproteins play a pivotal role in determining binding sites on the cell surface and in regulating interactions between other membrane proteins. Such transmembrane glycoproteins, which have polypeptide segments facing both sides of the lipid bilayer, are uniquely placed to sample the external environment of the cell and at the same time have portions of their polypeptide chains in close proximity to the protein components which are arranged along the inner surface of the membrane. In the case of the human red blood cell membrane, the two major glycoproteins, glycophorin A [1] and the so-called band 3 polypeptide [2] both have the molecular features to accommodate these dual functions.

Abbreviations: PMSF – phenylmethane sulfonyl fluoride, EDTA – ethylenediaminetetracetic acid, β-Me – β-mercaptoethanol, SDS – sodium dodecyl sulfate, MW – molecular weight, Bis – N,N'-methylene bisacrylamide, R_m – relative mobility, K_R – retardation coefficient, CBB – Coomassie Brilliant Blue, NTCB – 2-nitro-5-thiocyanobenzoic acid, DTT – dithiothreitol, RBC – red blood cell, TPCK – L-1-Tosylamide-2-phenylethylchloro-methyl ketone

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The evidence in support of the idea that the cytoplasmic segments of these transmembrane glycoproteins interact directly with a submembranous cytoskeletal network is largely indirect and can be summarized as follows. Incubating leaky red cell ghost membranes with multivalent antispectrin antibodies causes a significant shift in the topography of the sialoglycopeptide components [3]. This is most easily explained by assuming that shifts in the distribution of the spectrin network caused by the antispectrin antibodies result in a corresponding shift in the overlying (and associated) transmembrane glycoproteins. The same results can also be obtained more indirectly by incubating partially spectrin-depleted red cell ghosts in media buffered at pH 5.5, which is close to the isoelectric point of spectrin. This treatment causes the intramembranous particles to form irregular clumps [4, 5]. Since the latter are assumed to contain most, if not all, of the major transmembrane glycoproteins [6, 7] it is again reasoned that perturbation of the spectrin network causes corresponding perturbations in the distribution of the transmembrane glycoproteins, the simplest interpretation being that there are direct noncovalent connections between the cytoplasmic domains of the transmembrane proteins and the underlying spectrin network.

A recent and more direct attempt to demonstrate associations between the transmembrane glycoproteins and spectrin has been reported by Yu and Branton, who have shown that reconstituted liposomes containing the band 3 polypeptide are able to bind significant amounts of spectrin and band 5 (actin) [8], and these spectrin-actin-band 3 complexes also seem to respond to pH manipulations.

If we accept these data at face value, they provide provocative evidence that spectrin and the cytoplasmic segments of the major glycoproteins do indeed reside close together when all are organized as components in the membrane. The data fall short of providing us with any clear idea as to how spectrin attaches to the inner surface of the red cell membrane and what segments of the transmembrane glycoproteins it actually binds to.

It is obvious that further insight into this complex multiprotein system depends upon our knowing more about the chemical and molecular properties of the spectrin molecules. The purpose of this report is to summarize what is now known (or assumed) about spectrin and then to present some recent data on the substructure of human erythrocyte spectrin.

When spectrin was first described almost a decade ago [9], it was assumed on the basis of preliminary findings that the principal components were composed of two homogeneous high-molecular-weight polypeptide chains. Although the available evidence favored a two-chain model, it was certainly not compelling, and in fact when additional studies were carried out in different laboratories just the opposite interpretation was proposed. Three different groups of investigators reported that their purified spectrin preparations contained multiple N-terminal amino acid residues [10–12], and two laboratories reported that purified spectrin preparations were distinctly heterogeneous when analyzed by isoelectric focusing [13, 14]. Both sets of observations strongly favored the idea that spectrin prepared by essentially similar techniques was composed of distinctly different polypeptide chains all of which seemed to have roughly equivalent polypeptide chain length. Further studies on the properties of the spectrin preparations analyzed in nonionic detergents provided a somewhat paradoxical result in that each of the apparently heterogeneous bands of spectrin seemed to react with the same antispectrin antisera, suggesting that each of the heterogeneous forms of spectrin shared common antigenic determinants [14].

METHODS

Spectrin and actin were extracted from human erythrocyte membranes by incubating ghost membranes in 5 volumes of 0.1 mM EDTA (pH 9.5), 0.5 mM β-Me, 0.03 mM PMSF, at 37°C for 30 min. The membranes were removed by centrifugation at 30,000g for 45 min, and the supernatant was concentrated by isoelectric precipitation at pH 5.1 by titrating with 1 N HCl. After incubating on ice for 1 h, the precipitated spectrin were pelleted at 1,400g for 5 min. The precipitate was redissolved in small volumes of column buffer (0.1% SDS, 10 mM Tris-HCl (pH 9.0), 0.5 mM β-Me, 0.5 mM EDTA, 0.03 mM PMSF, 0.02% NaN_3) and applied on a Sepharose 4B column (5 × 90 cm) equilibrated in column buffer. The column was eluted at 50 ml/h, and the effluent was monitored by OD_{280} and SDS gel electrophoresis. Pure spectrin fractions were pooled and dialyzed exhaustively against 2 mM Na phosphate (pH 9.0), 0.5 mM β-Me, 0.5 mM EDTA, 0.03 mM PMSF, 0.02%

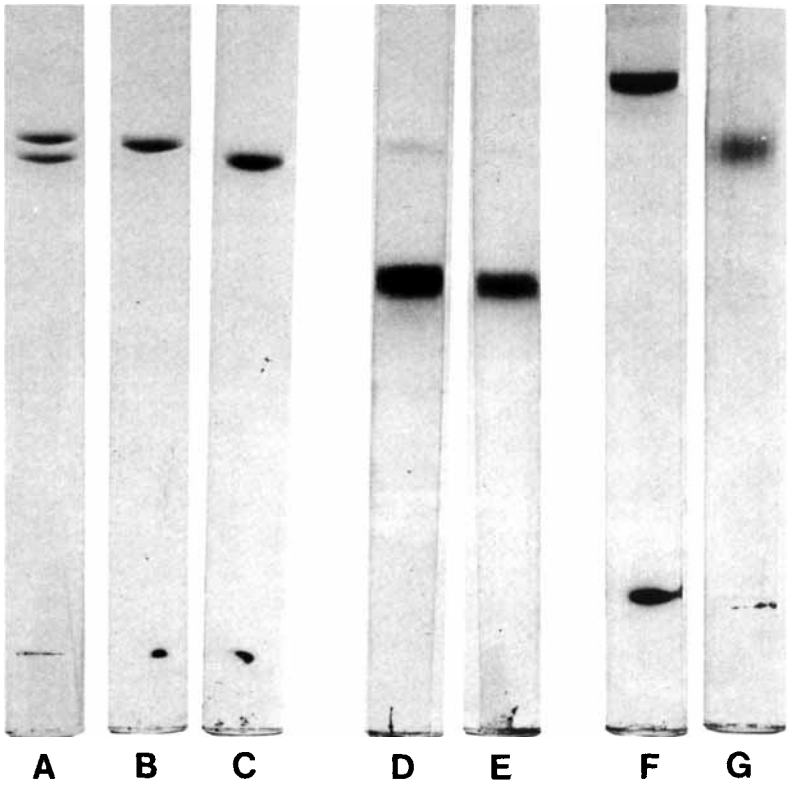

Fig. 1. Electrophoretic and isoelectric focusing analysis of different spectrin preparations. Purified spectrin (A), isolated band 1 (B), and isolated band 2 (C) on 4% SDS gels (Fairbanks); purified spectrin (D) and isolated band 1 (E) analyzed by isoelectric focusing gels containing pH 3–7 ampholytes with 8 M urea (pI of band 1 is 5.5); isolated band 1 (F) and isolated band 2 (G) on 5.5% gels run in Tris-glycine buffer, pH 8.9 (Ornstein and Davis).

NaN_3 at 4°C. After removal of SDS, spectrin solution was concentrated to 1 mg/ml in an Amicon cell with XM-100 membrane and stored at 0°C.

In 0.1% SDS, spectrin was dissociated and fractionated by subunit size. Subunit band 1 can be obtained by rechromatography of the leading edge of the peak. Spectrin subunit band 2 was purified by preparative SDS gel electrophoresis on 4% acrylamide Fairbank's gel (0.6 × 9 × 33 cm). Purified spectrin (2 mg) in 10 mM Na borate (pH 9.0) was reacted with 0.1 mg fluorescamine (Roche) in 2 μl acetone, and 4 mg unlabeled spectrin was added to the labeled spectrin. The mixture (~ 4 ml) was made to 20 mM Tris-HCl (pH 7.5), 3% SDS, 1.5% β-Me, 2 M urea, with a small amount of Bromophenol Blue added. The protein was applied on the slab gel and electrophoresed at 50 mA for 36 h. Band 2 was excised from the gel under a UV lamp and protein was eluted from the pulverized gel in column buffer. SDS was removed by prolonged dialysis against the buffer used for pure spectrin.

Isoelectric Focusing

Isoelectric focusing was done on acrylamide tube gel matrix, according to the method of Bhakdi et al [13]. The gels (0.5 × 10 cm casted in glass tubes) contained 4% acrylamide cross-linked with 2.5% Bis, 10% sucrose, 8 M urea, and 1% ampholines (pH range 3.5–7.0 or 3.5–10.0). The polymerization was catalyzed by the addition of tetramethylethylenediamine and ammonium persulfate. The anodal buffer (0.03 M NaOH) and cathodal buffer (0.05 M H_2SO_4) were thoroughly degased and the gels were preelectrophoresed at 1 mA/gel for 15 min to remove persulfate. Samples containing 10 μg protein in 8 M urea were applied on top of the gel, protected by a 0.5-cm layer of 0.1% ampholine in 5% sucrose. Current at 0.5 mA/gel was applied during the first 5 h of electrofocusing and then changed to constant voltage of 40 V/cm for another 12 h. The final current had dropped to 0.1 mA/gel. The gels were fixed in 25% 2-propanol after two changes to completely remove ampholine, then stained in 0.05% CBB in 25% 2-propanol for 5 h, then in 0.0025% CBB in 25% 2-propanol for 16 h.

Molecular Weight Determination

The molecular weights of the spectrin bands 1 and 2 were determined by Ferguson plot [19] in the Ornstein [36] and Davis [37] gel system. The gels (0.5 × 11 cm casted in glass tubes) contained 3.0, 3.75, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.5, and 9.0% acrylamide with constant Bis:acrylamide = 1:30, in 3.75 mM Tris-HCl (pH 8.9). Electrode buffer was Tris-glycine buffer containing 25 mM Tris, 0.2 M glycine (pH 8.9). Samples contained 10–25 μg protein in 50% sucrose Tris-glycine buffer with Bromphenol Blue as tracking dye. Electrophoresis was run at 4 mA/gel for 3 h, and the gels were stained in 0.05% Coomassie Brilliant Blue. The relative mobility R_m was measured in different percentage acrylamide gels and the values of log (R_m × 100) versus percentage acrylamide concentrations T were plotted (Fig. 2). According to the Ferguson equation:

$$\log R_m = -K_R T + \log Y_0$$

The retardation coefficient, K_R, is proportional to molecular weight at constant Bis/acrylamide ratio. The free mobility $Y_0 = R_m$ at T = 0. Linear regression analyses were made on all Ferguson plots to determine K_R and Y_0. K_R of molecular weight protein standards were also determined on identical gel systems to construct the molecular weight standard plot (Fig. 3) [ie, plot K_R versus molecular weight]. The molecular weight of spectrin bands 1 and 2 were obtained by interpolation on the molecular weight standard curve.

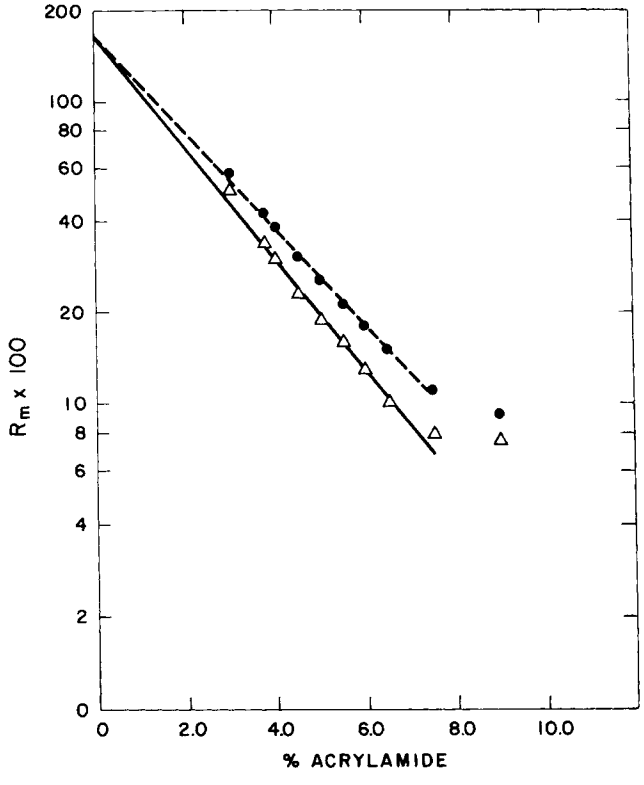

Fig. 2. Ferguson plot of spectrin band I (△——△) and band II (●– – –●). Relative mobilities R_m were measured as described in Methods. The logarithm of $R_m \times 100$ versus the gel acrylamide concentration were plotted. Linear regression analysis results: for band 1 $K_R = -0.157$, for band 2 $K_R = -0.140$.

NTCB Cleavage

Purified spectrin or subunits band 1 or band 2 (1–3 mg) was reduced with 10 mM DTT in 6 M guanidine-HCl (pH 8.0), 1 mM EDTA. After reduction, DTT was removed by dialysis against 100 vol 8 M urea, 1 mM EDTA followed by two more dialyses against the cleavage reaction medium (8 M urea, 0.2 M Tris-HCl (pH 8.0), 1 mM EDTA). Sulfhydryl groups were cyanylated by a two-fold excess of NTCB as described by Jacobson et al [21]. Hydrolysis was achieved by adjusting the pH to 9.0 and incubating over night at 37°C. After cleavage, 10 μl of β-Me per milliliter of reaction mixture was added and incubated for 20–30 min. The reaction mixture was dialyzed twice against 1 mM Tris base, 2 M urea, 0.1 M NaCl, then against 1 mM Tris base. Finally, the reaction mixture was dialyzed against distilled water and lyophilized. The cleavage proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis. The first dimension was run on 5% alkaline urea gel [36, 37] and the second dimension was run on 7.5% acrylamide Fairbank's slab gel.

Amino acid analysis was performed on a Durrum-D500 amino acid autoanalyzer. Determinations on three different preparations of spectrin and subunits gave similar results. Partial tryptic cleavage of spectrin subunits are described in the figure legend of Figure 4.

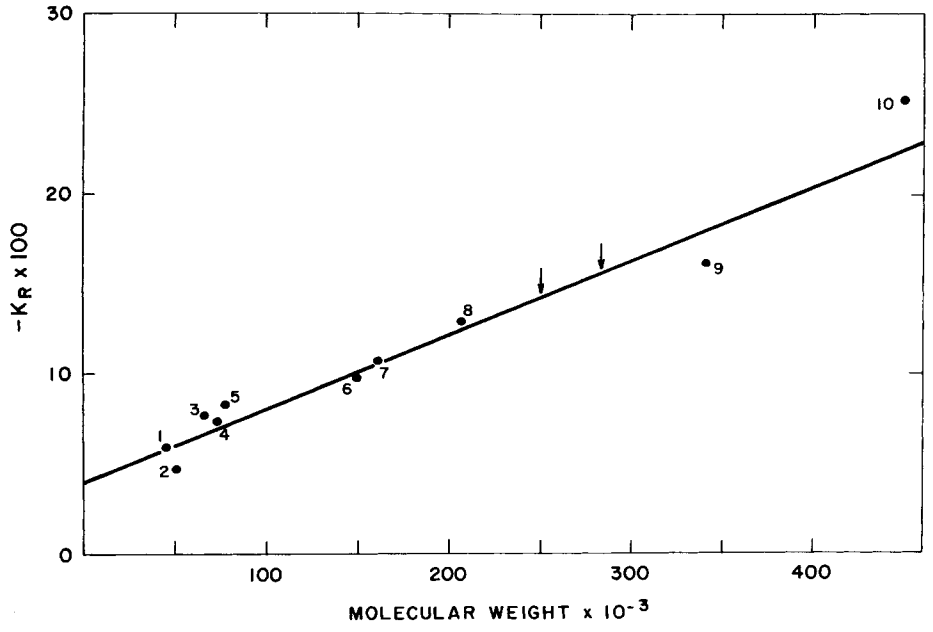

Fig. 3. The retardation coefficient vs molecular weight standard curve. The retardation coefficient K_R was obtained as described in Methods. The proteins used are: 1) Ovalbumin, 45,000; 2) hog pancreas α-amylase, 50,000; 3) bovine albumin, 66,200; 4) lactate dehydrogenase (lactobacillus leichmanni), 72,000 (monomer); 5) human transferrin, 76,500; 6) rabbit muscle aldolase, 149,000; 7) sheep IgG, 160,000; 8) sweet potato β-amylase, 206,000; 9) bovine fibrinogen, 340,000; 10) Apo ferritin, 450,000. Linear regression analysis yielded the following results: slope = −0.0043551, Y intercept = −0.0384, standard deviation of Y = 0.061, correlation coefficient of the regression = −0.9796. K_R of bands I and II were the averages of three determinations as described in Figure 2. The molecular weights obtained by interpolation on the standard curve are band I = 273,500 and band II = 238,600.

RESULTS

We have recently reinvestigated the question of the homogeneity of the spectrin polypeptides by analyzing spectrin purified by both a high-salt gel filtration procedure [15] and by purifying different spectrin polypeptides by gel chromatography and/or preparative gel electrophoresis in the presence of sodium dodecyl sulfate [15]. Purified fractions of spectrin band 1 and spectrin band 2 can be prepared by preparative SDS chromatography, in the case of band 1, or by preparative SDS gel electrophoresis, in the case of band 2 (Fig. 1). In the course of these studies we have never found any indication that the high-molecular-weight polypeptides of spectrin are aggregated forms of low-molecular-weight polypeptides. We also feel that the previous reports which describe low-molecular-weight forms of spectrin polypeptides are undoubtedly due to artifactual proteolytic digestion of the spectrins either from contaminating leukocytes and/or platelets or the action of proteases bound to the red cell membrane itself [16–18]. Spectrin prepared by the high-salt procedure has a tendency to break down slowly during storage at 4°C, while spectrin prepared by SDS chromatography remains stable for long periods of time at 4° and also retains its capacity to bind to inside-out red cell membrane vesicles [15].

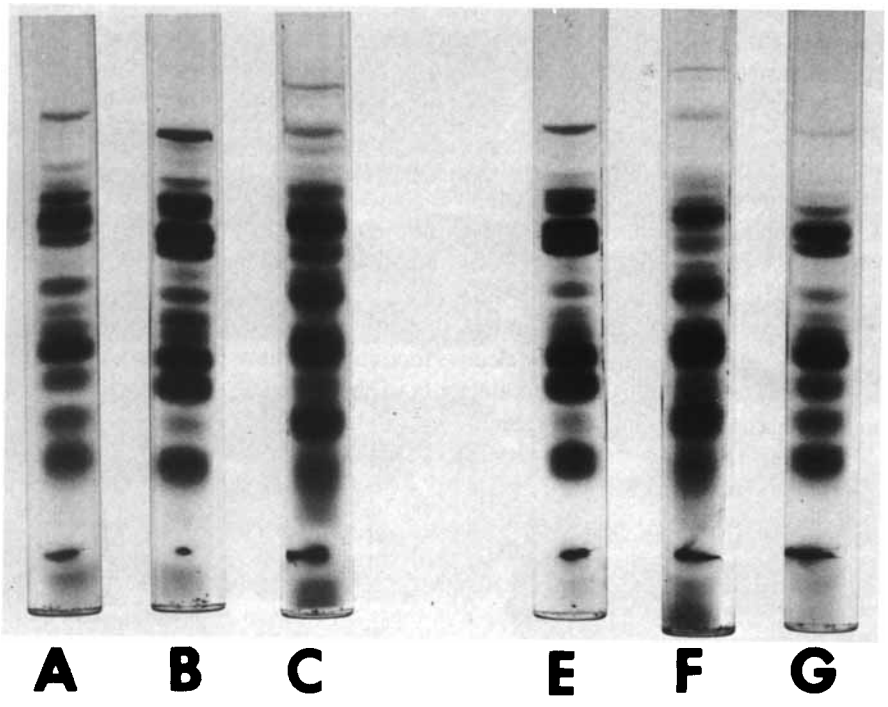

Fig. 4. Partial tryptic digestion of spectrin subunits. The spectrin subunits band I, band II, and unfractionated spectrin (Ca 0.5 mg/ml in 20 mM Tris-HCl, pH = 8.0) were digested with TPCK-trypsin (enzyme:substrate = 1:100) at 0°C, 50 μl aliquots were taken at 15-min intervals, and the digestion was stopped by adding 50 μl boiling SDS solubilizing buffer, following which the mixtures were boiled for 2 min. The samples were analyzed on 5.6% Fairbanks SDS gel system. A) band I, B) band II, C) band I digested for 60 min at 0°C.

Spectrin purified in the presence of SDS also has another interesting property. When spectrin is dissolved in SDS its circular dichroism (CD) spectrum is significantly altered, and a relatively large amount of α-helicity is lost [15]. However, when the SDS is dialyzed away from such preparations the α-helical content of the spectrin molecules is essentially restored, and the CD spectra of SDS-purified spectrin are indistinguishable from the spectra of spectrin purified by conventional high-salt treatment. As mentioned above, SDS-purified spectrin also binds as efficiently to inside-out red cell vesicles as spectrin prepared in the absence of detergents [15].

The ability of spectrin to regain tertiary structure after SDS treatment is surprising, since it is such an enormous polypeptide chain. It is possible that some parts of the molecule, not part of the helical segments or the membrane-binding sites, are irreversibly altered by the SDS treatment. However, the ability to purify spectrin in SDS offers certain operational advantages, in that SDS chromatography is probably the best way to prepare spectrin free of proteases, and is the only way we have been able to purify the individual subunits.

We have also studied the isoelectric focusing properties of spectrin purified in the presence of SDS and have obtained results which differ significantly from previously pub-

lished reports. SDS-purified band 1 of spectrin forms a reasonably homogeneous band on isoelectric focusing in acrylamide gels (Fig. 1), which is distinctly more focused than bands obtained by previous investigators [13, 14]. SDS-purified band 2 also forms a single band but the latter is more diffuse and less properly focused than the band 1 subunit. In order to explain the discrepancy between our results and those of previous investigators, we have studied the focusing properties of spectrin in some detail and have discovered that the capacity to achieve well-focused bands depends upon the amount of spectrin polypeptide which is applied to the gel. Relatively small amounts of the individual spectrin peptides focus as narrow bands, but if increasing amounts of the same material are applied to different gels, the resulting bands become increasingly broad. Thus we feel that the very broad bands previously achieved by isoelectric focusing may have been due to excessive sample loads and/or secondary aggregation states of the spectrin polymers which take place even in the presence of nonionic detergents.

Further evidence in support of spectrin homogeneity has been obtained by analyzing purified spectrin subunits by standard gel electrophoresis without SDS or other denaturing agents. SDS-purified band 1 forms remarkably sharp bands (Fig. 1); SDS-purified band 2 forms a band which is more diffuse and is reminiscent of the diffuse band achieved when the latter polypeptide is analyzed by isoelectric focusing. It is interesting that the isolated individual bands of spectrin can be analyzed in these gel systems even though the complete spectrin molecule, composed of equal amounts of bands 1 and bands 2, does not migrate into these gels without denaturing agents.

We have taken advantage of this ability to electrophorese the spectrin subunits under these conditions to reinvestigate their molecular weights using the Ferguson method of analysis, which is based on free-mobility calculations [19]. Plots of the mobilities of a set of standard proteins are illustrated in Fig. 3, and the plots obtained when purified band 1 and purified band 2 are analyzed under the same conditions are shown in Fig. 2. Molecular weights of spectrin subunits calculated by this approach give values of 273,500 for band 1 and 238,600 for band 2, which are in remarkably good agreement with the molecular weights calculated using the standard SDS relative mobility method, a result which represents a welcome confirmation of the latter procedure.

The results of analyzing purified spectrin subunits by SDS gel electrophoresis, isoelectric focusing, and standard gel electrophoresis all support the original claims that the spectrin polymers represent homogeneous high-molecular-weight polypeptides. These findings do not support more recent suggestions that spectrin represents a heterogeneous mixture of chemically different proteins. However, it should be realized that the evidence in favor of spectrin being composed of two homogeneous chains is not as rigorous as one would like, and it must be admitted that some heterogeneity in the polypeptide chains could easily pass undetected by these studies. We also have not provided an entirely satisfactory explanation for the multiple N-terminal residues reported by others [10, 12]. It is conceivable that the N-terminal analysis of such mammoth polypeptides could be misleading, particularly since quantitative recoveries of terminal amino acids have not been obtained. It is also conceivable that some of the amino acids detected in these studies represent those exposed by proteolytic cleavage of the spectrin polypeptides. The latter possibility must be considered in the face of clear evidence that spectrin peptides are extremely sensitive to such degradation [20].

Is the smaller subunit of spectrin (band 2) simply a shorter version of band 1?

The amino acid compositions of SDS-purified spectrin band 1 and SDS-purified band 2 are similar (Table I), although there are differences in individual residues. A more revealing attempt to determine the degree of molecular overlap between the two bands has been provided by study of the peptide fragments of the two forms produced by two different methods. Purified band 1 and band 2 have been subjected to tryptic cleavage for different time periods, and the resulting peptide fragments analyzed by SDS gel electrophoresis. Figure 4 shows the electroferograms obtained when both peptides are digested at 30 and 60 min. Although these comparative maps are complex and not easy to interpret, it seems clear that the major Coomassie Blue staining peptides generated by tryptic cleavage from each of the spectrin subunits differ significantly in their SDS mobility. Peptide differences are evident after both 30 and 60 min of cleavage and thus indicate that there are significant differences in the location of lysine and arginine residues in the two polypeptide chains. Comparative studies of peptides generated from both spectrin subunits by S-cyanylation have also been carried out, with comparable results. Figure 5 shows two-dimensional electroferograms of S-cyanylation-induced fragments of both SDS-purified band 1 and SDS-purified band 2. Since S-cyanylation cleaves peptide bonds under relatively mild conditions at sites of cystine linkages [21], the different peptide maps obtained from the two peptides further reinforce the idea that spectrin band 1 and spectrin band 2 represent significantly different primary sequences. The cleavage maps prepared by S-cyanylation of the spectrin subunits were carried out in urea or guanidine; thus this method of generating peptides is not influenced by the conformational states of the different peptides. One drawback of the S-cyanylation procedure evident here and in other studies is the fact that such cleavages are often incomplete. We assume that this explains the presence of a relatively large number of high-molecular-weight peptides present in both maps.

TABLE I. Amino Acid Composition of Human Spectrin Subunits (in mole %)

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

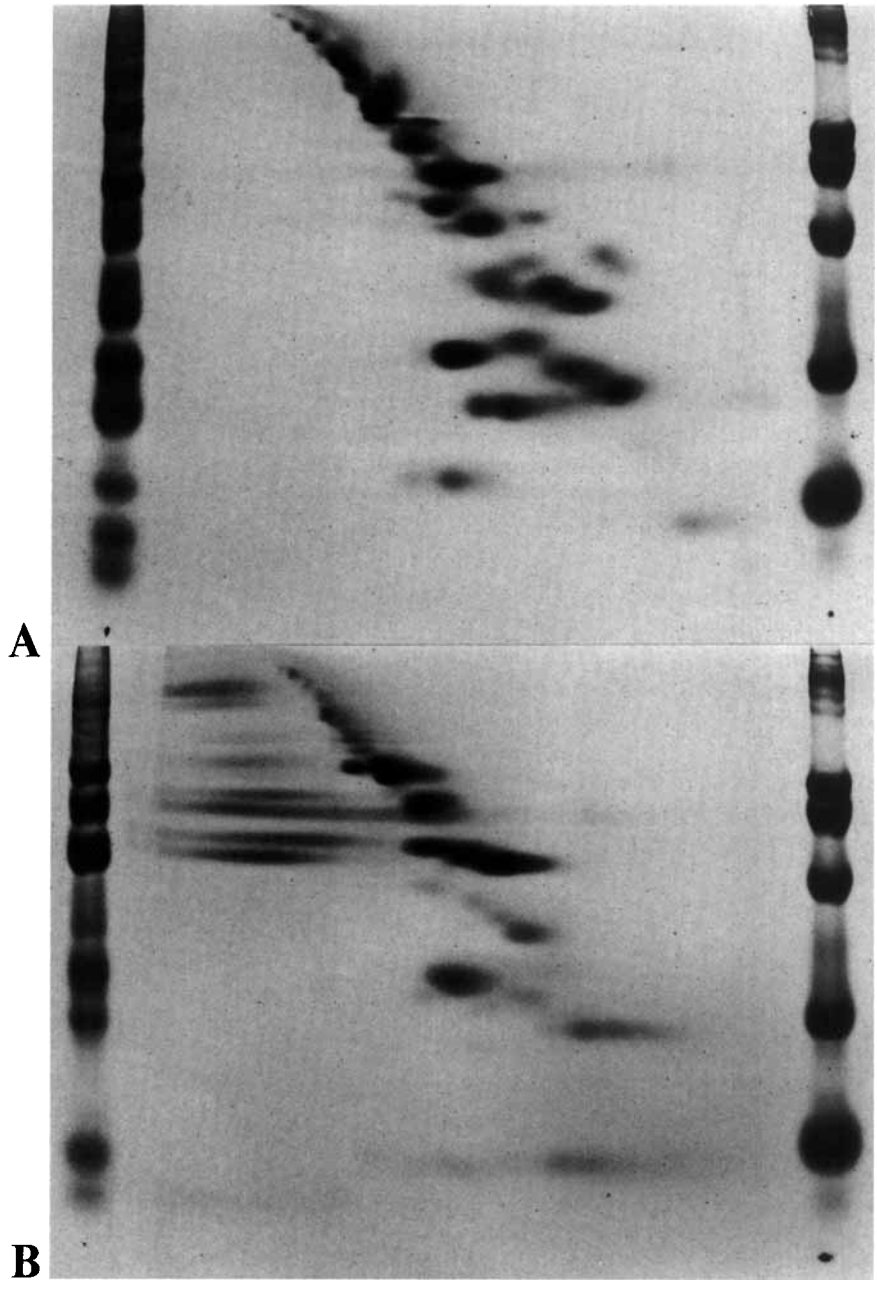

Fig. 5. Mapping of NTCB-cleaved spectrin bands 1 and 2. First 250 μg of each cleaved polypeptide was separated in a 5% polyacrylamide gel in 8 M urea (pH 8.9), and the second dimension was run on a 7.5% acrylamide slab as described under Methods; 150 μg of material and 25 μg of markers were electrophoresed in the lateral slots as controls. Markers used: transferrin (90,000 MW), human albumin (67,000 MW), ovalbumin (45,000 MW), chymotrypsinogen A (23,200 MW), and human hemoglobin (16,000 MW). A) NTCB-cleaved spectrin band 1; B) NTCB-cleaved spectrin band 2.

SUMMARY AND CONCLUSION

Our understanding of the substructure of the spectrin polypeptides is still at a rudimentary stage, but it is possible to draw some tentative conclusions with the evidence at hand. Spectrin bands 1 and 2, which have apparent molecular weights of 250,000 and 225,000 daltons, seem to be composed of single polypeptide chains. The evidence in support of this conclusion is not unequivocal but rests more on the fact that evidence of heterogeneity is less convincing. On the basis of partial enzymatic and chemical cleavage studies, it appears that spectrin band 1, the higher-molecular-weight form, differs significantly in its primary structure from spectrin band 2. Thus spectrin band 2 is not simply a shorter version of the band 1 polypeptide. The evidence suggesting significant differences in amino acid sequence of the two spectrin peptides is amplified by the often repeated observation that only the band 2 of spectrin is phosphorylated [22] and the recent observation that only isolated spectrin band 2 binds to inside-out vesicles [15]. Although the chemical and enzymatic cleavage experiments suggest significant differences in primary structure between the two subunits, it is important to point out that there probably are also large areas of overlap between the two. This conclusion is based on the fact that the isolated components have similar amino acid compositions and closed isoelectric focusing points, and both peptides show significant immunologic cross-reactivity.

Earlier cross-linking studies suggested that the isolated spectrin molecule was made up of heterodimers of bands 1 and 2 [23], and spectrin also appears to be a heterodimer when organized in the intact ghost membrane [24]. Recent immunocytochemical studies indicate that spectrin is localized as a submembranous matrix along the inner surfaces of intact human blood cells [25]. These studies confirm earlier suggestions that the spectrin is located internal to the lipid bilayer of erythrocyte ghosts, as determined both by ferritin-antibody localization [26] and by its inaccessibility to enzymatic digestion or to exogenous chemical labeling [27, 28].

The precise three-dimensional disposition of the spectrin matrix within intact red blood cells is still incompletely understood. Earlier conceptions of the spectrin molecule suggested that it might be myosin-like in form – and idea based on its hydrodynamic properties [29] and by an apparent cross-reactivity between spectrin and muscle myosin [30]. This idea led many to suggest that spectrin might form a submembranous reticulum, perhaps attached to cytoplasmic segments of transmembrane proteins or to other membrane elements. Such a reticulum was thought to confer stability and deformability to the cell membrane. One of the most convincing findings in support of a spectrin network has been provided by analyzing RBC ghost residues prepared by Triton extraction [31]. Triton selectively solubilizes integral membrane proteins and some of the membrane lipids, and it leaves behind a reticular network which still retains the approximate size and shape of the original membrane. Although the exact composition of this network remains undefined, it is reasonable to suppose that its principal components are spectrin and band 5, the putative erythrocyte actin.

These studies illustrate the remarkable stability of spectrin-actin complexes, but leave unanswered how spectrin and actin interact in the intact red cell membrane. Another still unresolved general problem concerns the attachment sites for the submembranous spectrin scaffolding. Earlier studies suggested that spectrin might associate directly with the cytoplasmic segments of at least two transmembrane glycoproteins [3–6], but recent

experiments do not support these ideas [15, 32]. The results of binding studies from our laboratory, which are to be reported elsewhere [15], indicate that the spectrin polypeptides bind to specific regions on the inner surface of the red cell membrane which are in close proximity to the cytoplasmic segments of band 3 and to bands 4.1–4.2. We have also shown that spectrin can form specific complexes with isolated bands 4.1–4.2. Although spectrin does not appear to bind directly the cytoplasmic segment of band 3 [32], antibodies to this segment can compete for spectrin binding to inside-out vesicles [15]. Purified spectrin does bind to isolated bands 4.1-4.2 [33] and antibodies to the bands 4.1–4.2 complex also inhibit spectrin binding to the inner surface of the ghost membrane [15]. The close associations between spectrin and band 4.1 suggested by these findings is consistent with earlier cross-linking studies [24] and the recent demonstration that a calcium activated transglutaminase in red cells catalyzes the formation of specific complexes between spectrin and band 4.1 and possibly band 3 as well [34, 35].

The studies to date which have attempted to define the site or sites of spectrin binding to the inner surface of the red cell membrane suggest that spectrin binding sites are concentrated at specific loci which are clustered around or perhaps defined by cytoplasmic segments of the major transmembrane glycoproteins. It is obvious, however, that that available information provides us with only a hazy glimpse of what will undoubtedly prove to be highly complex associations between the spectrin polypeptides and various elements of the red cell membrane.

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REFERENCES

1. Tomita M, Marchesi VT: Proc Natl Acad Sci USA 72:2964, 1975.
2. Steck TL, Ramos B, Stapazon E: Biochemistry 15:1154, 1976.
3. Nicolson GL, Painter RG: J Cell Biol 59:395, 1973.
4. Pinto da Silva P: J Cell Biol 53:777, 1972.
5. Elgsaeter A, Branton D: J Cell Biol 63:1018, 1974.
6. Pinto da Silva P, Nicolson GL: Biochim Biophys Acta 363:311, 1974.
7. Shotton D, Thompson K, Wofsy L, Branton D: J Cell Biol 76:512, 1978.
8. Yu J, Branton D: Proc Natl Acad Sci USA 73:3891, 1976.
9. Marchesi VT, Steers EJ: Science 159:203, 1968.
10. Knufermann H, Bhakdi S, Schmidt-Ullrich R, Wallach DFH: Biochim Biophys Acta 330:356, 1973.
11. Fuller GM, Boughter JM, Morazzani M: Biochemistry 13:3036, 1974.
12. Dunn MJ, Maddy AH: FEBS Lett 36:79, 1973.
13. Bhakdi S, Knufermann H, Wallach DFH: Biochim Biophys Acta 345:448, 1974.
14. Green JR, Dunn MF, Spooner RL, Maddy AH: Biochim Biophys Acta 373:51, 1974.
15. Litman D, Hsu CJ, Marchesi VT: Manuscript submitted.
16. Morrison WL, Neurath H: J Biol Chem 200:39, 1953.
17. Bernacki RJ, Bosmann HB: J Membrane Biol 7:1, 1972.
18. Tokes ZA, Chambers SM: Biochim Biophys Acta 389:325, 1975.
19. Ferguson KA: Metabolism 13:985, 1964.
20. Hulla FN: Biochim Biophys Acta 345:430, 1974.
21. Jacobson GR, Schaffer MH, Stark GR, Vanaman TC: J Biol Chem 248:6583, 1973.

22. Birchmeier W, Singer SJ: J Cell Biol 73:647, 1977.
23. Clarke M: Biochem Biophys Res Commun 45:1063, 1971.
24. Wang K, Richards FM: J Biol Chem 249:8005, 1974.
25. Ziparo E, Lemay A, Marchesi VT: Manuscript submitted.
26. Nicolson GL, Marchesi VT, Singer SJ: J Cell Biol 51:265, 1971.
27. Marchesi VT: in Fox CF (ed): "Biochemistry of Cell Walls and Membranes." MTP International Review of Science–Biochemistry Series 1, Volume 2. Baltimore: University Park Press, 1975.
28. Kirkpatrick FH: Life Sci 19:1, 1976.
29. Schechter NM, Sharp M, Reynolds JA, Tanford JA, Tanford C: Biochemistry 15: 1897, 1976.
30. Sheetz MP, Painter RG, Singer SJ: Biochemistry 15:4486, 1976.
31. Hainfeld JF, Steck TL: J Supramol Struct 6:301, 1977.
32. Bennett V, Branton D: J Biol Chem 252:2753, 1977.
33. Hsu CJ, Litman D, Marchesi VT: Manuscript in preparation.
34. Lorand L. Weissmann LB, Epel DL, Bruner-Lorand J: Proc Natl Acad Sci USA 73:4479, 1976.
35. Anderson DR, Davis JL, Carraway KL: J Biol Chem 252:6617, 1977.
36. Ornstein L: Ann NY Acad Sci 121:321, 1964.
37. Davis BJ: Ann NY Acad Sci 121:404, 1964.