Control of Erythrocyte Membrane-Skeletal Cohesion by the Spectrin-Membrane Linkage[†]

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ABSTRACT: Spectrin tetramer is the major structural member of the membrane-associated skeletal network of red cells. We show here that disruption of the spectrin−ankyrin−band 3 link to the membrane leads to dissociation of a large proportion of the tetramers into dimers. Noncovalent perturbation of the linkage was induced by a peptide containing the ankyrin-binding site of the spectrin β-chain, and covalent perturbation by treatment with the thiol reagent, *N*-ethylmaleimide (NEM). This reagent left the intrinsic self-association capacity of the spectrin dimers unaffected and disturbed only the ankyrin−band 3 interaction. The dissociation of spectrin tetramers on the membrane into functional dimers was confirmed by the binding of a spectrin peptide directed against the self-association sites. Dissociation of the tetramers resulted, we infer, from detachment of the proximal ends of the constituent dimers from the membrane, thereby reducing their proximity to one another and thus weakening their association. The measured affinity of the interaction of the peptides with the free dimer ends on the membrane permits an estimate of the equilibrium between intact and dissociated tetramers on the native membrane. This indicates that in the physiological state the equilibrium proportion of the dissociated tetramers may be as high as 5−10%. These findings enabled us to identify an additional important functional role for the spectrin−ankyrin−band 3 link in regulating spectrin self-association in the red cell membrane.

The shear elasticity of the red cell membrane and its exceptional resistance to mechanical stress are independently regulated properties (*1*), yet both are wholly dependent on the proteins of the membrane skeletal network. This consists of long elastomeric spectrin molecules, predominantly in the form of tetramers, attached at their distal ends to nodes, or junctions, made up of actin, 4.1R, and other proteins (for a review, see ref *2*). The shear elasticity of the membrane is thought to be primarily regulated by extension of the spectrin tetramers and reversible unfolding of certain of their constituent triple-helical repeats, while the maintenance of normal membrane mechanical stability depends on the tetrameric state of spectrin (*1, 3, 4*). Weakened spectrin self-association due to mutations in α- or β-spectrin leads to reduced membrane mechanical stability and fragmentation of the cell (*2*). It appears paradoxical that the apparent dissociation of tetramers into their constituent dimers, with consequent interruption of the continuity of the network, as occurs when red cells are treated with the thiol-directed reagent, *N*-ethylmaleimide (NEM),[1] not only reduces the membrane mechanical stability but also increases the stiffness of the membrane (*1, 5, 6*). A further unexplained feature of the effect of NEM on the cells is that, regardless of the NEM concentration and the time of reaction, the proportion of spectrin dissociated into dimers does not exceed 70−75% (*4*). We have investigated these phenomena, and the results appear to indicate the need for a re-examination of the structural basis of spectrin self-association and its relation to membrane elasticity.

MATERIALS AND METHODS

Reagents and Antibodies. Polyclonal antibodies against ankyrin and band 3 were generated in our laboratory. Unless otherwise indicated, all other reagents were purchased from Sigma Aldrich (St. Louis, MO).

Cells. Fresh blood was taken with informed consent from healthy volunteers by venipuncture, with EDTA as the anticoagulant. After the buffy coat had been discarded, the red cells were washed three times with PBS [137 mM NaCl, 10 mM sodium phosphate, and 2.7 mM KCl (pH 7.4)] and resuspended in PBS.

Microscopy. Cells and resealed ghosts, sampled before and after manipulations involving reagents, were suspended at 10% hematocrit in PBS, supplemented with 0.1% (w/v) bovine serum albumin (BSA), and examined by phase-contrast microscopy.

Peptides. The preparation and properties of the peptides corresponding to the sites of interaction of α- and β-chains in forming the tetramer from two dimers of both spectrin I (erythroid) and II (nonerythroid) have been previously described (*7, 8*). A spectrin fragment corresponding to structural repeats 14 and 15 of the spectrin I β-chain, which contains the ankyrin-binding site, was subcloned into the pGEX-4T-2 vector using EcoRI and SalI restriction enzymes upstream and downstream, respectively. The fidelity of the construct was confirmed by DNA sequencing. All the recombinant fragments were purified on a glutathione-Sepharose 4B affinity column.

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[1]Abbreviations: NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DI, deformability index; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; IOVs, inside-out vesicles.

Reaction with NEM. Red cells at 10% hematocrit were treated with NEM at concentrations of up to 2 mM for 15 min at 37 °C. In other experiments, the modification was conducted on ghosts, prepared by lysis of washed cells in 35 volumes of ice-cold hypotonic buffer A [5 mM Tris and 5 mM potassium chloride (pH 7.4)], followed by resuspension in the same buffer and centrifugation for 10 min at 20000*g* until the ghosts became white. Isotonicity was then restored by addition of 50 mM Tris and 1.5 M potassium chloride (pH 7.4), before the treatment with up to 2 mM NEM for 1 h at 4 °C. The reaction was terminated by incubation with 10 mM dithiothreitol (DTT) for 15 min at 37 °C (intact cells) or 2 min at 4 °C (ghosts).

Binding of Peptides. The procedure followed was that of An et al. (*7*). Peptides αI(1−154), αII(1−149), βI(15-C), and βIR-(14−15) at a series of concentrations were each added to hypotonic ghosts on ice; these ghosts were prepared by lysis in buffer A, containing in addition 1 mM Mg-ATP. Resealing was effected by restoration of tonicity and incubation at 37 °C. After being washed to remove the external peptide, the sealed ghosts were incubated at the desired temperature for 40 min. The amount of bound peptide was determined by extraction of the total spectrin as described below, followed by electrophoresis under nondenaturing conditions in a 5% polyacrylamide gel in a Tris-Bicine buffer system in the cold (*9*). The relative concentrations of the spectrin dimer−peptide complex and spectrin tetramer were evaluated by staining and densitometry, using ImageJ (available from the National Institutes of Health).

Ektacytometry. Modified and control ghosts were suspended in 40% dextran (M_w = 40000 kDa), and fragmentation under shear as a function of time was assessed in the ektacytometer (*10*). The membrane stability was expressed as the rate of decrease of the deformability index (DI) at a constant applied shear stress of 750 dyn/cm^2.

Extraction of Spectrin. Ghosts prepared by lysis of red cells in ice-cold hypotonic buffer A were twice washed in the same buffer, and twice more with extraction medium {buffer B [0.25 mM sodium phosphate (pH 7.4)]}. Spectrin was extracted by overnight dialysis at 4 °C in buffer B and recovered in the supernatant after centrifugation for 20 min at 20000*g*. The protein concentration was determined spectrophotometrically, taking *E*(280 nm; 1 mg/mL; 1 cm) to be 1.07.

Analysis of the Dimer−Tetramer Equilibrium of Modified Spectrin in Solution. Total extraction of spectrin was performed by incubation of ghosts with 40 volumes of 0.5 mM EDTA for 30 min at 37 °C, followed by centrifugation as described above. The resulting spectrin dimer was made isotonic and allowed to react with NEM at a series of concentrations for 1 h at 37 °C. Excess reagent was quenched by addition of 10 mM DTT. The modified spectrin samples thus generated were incubated at 30 °C for 4 h to allow the system to reach the self-association equilibrium. The relative concentrations of dimer and tetramer were determined by polyacrylamide gel electrophoresis in the cold as described above.

Preparation and Modification of Membrane Skeletons. Ghosts were incubated in 20 volumes of buffer C [625 mM sodium chloride, 6.25 mM sodium phosphate, 0.625 mM EGTA, and 0.625 mM DTT (pH 7.5)] with or without 6% (v/v) Triton X-100 for 30 min at 4 °C. The resulting membrane skeletons were collected by centrifugation (20000*g* for 20 min) and washed by resuspension in PBS. They were then incubated in buffer A with or without NEM, resuspended after centrifugation in an equal volume of Tris-glycine gel electrophoresis buffer containing 10%

SDS (*11*), heated, and applied to a 10% SDS−polyacrylamide gel in the same buffer.

Extraction of Ankyrin from Spectrin-Depleted Inside-Out Vesicles of Modified Ghosts. Inside-out vesicles (IOVs) from ghosts before and after NEM treatment were prepared by extraction of spectrin in buffer C at 37 °C, as described above. For extraction of ankyrin, the IOVs were incubated for 30 min at 37 °C in 40 volumes of solutions containing potassium iodide at concentrations of up to 1 M in buffer D [10 mM sodium phosphate, 1 mM EDTA, 1 mM DTT, 1 mM diisopropyl fluorophosphate (DFP), and 0.02% (v/v) Tween 20]. After centrifugation for 30 min at 20000*g*, the IOVs were washed by suspension in buffer D, dissolved in electrophoresis sample buffer as described above, and subjected to electrophoresis in a 10% SDS−polyacrylamide gel. The separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), which was blocked for 1 h in PBS-T [137 mM NaCl, 10 mM sodium phosphate, 2.7 mM KCl, and 0.1% Tween 20 (pH 7.4)] containing 4% (w/v) nonfat milk powder and 1% (w/v) bovine serum albumin (BSA), followed by incubation for 1 h at room temperature with anti-ankyrin or anti-band 3 antibody. Blots were then washed three times with PBS-T and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit antibody. Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) method (Pierce, Rockford, IL). Bands were quantified with the aid of ImageJ.

Extraction of Band 3. To eliminate the extracellular glycosyl chains from band 3, red cells were washed three times with PBS, resuspended at 50% hematocrit in PBS, and incubated with 1 mg/mL chymotrypsin for 1 h at 37 °C. The enzyme was inactivated by addition of Protease Inhibitor Cocktail. NEM treatment was performed for 15 min at 37 °C, as described above, and the cells were then lysed in buffer A and washed in the same buffer until they became white. Band 3 was extracted, following the protocol of Bennett et al. (*12*). The ghosts were resuspended in buffer E [100 mM potassium chloride, 10 mM sodium phosphate, 1 mM EDTA, 0.2 mM DTT, and 1 mM DFP (pH 7.5)] with or without Triton X-100 for 15 min at 4 °C. After centrifugation at 40000*g* for 15 min, the samples were washed with buffer D, resuspended in an equal volume of electrophoresis buffer (*11*), and applied to a 10% SDS−polyacrylamide gel. Western blots of these gels were analyzed as described above for relative concentrations of band 3 and ankyrin, normalized to that of actin.

Estimation of Lipid Asymmetry. To assay for exposure of phosphatidylserine in the outer leaflet of the intact cell membrane, cells were incubated with fluorescein-conjugated annexin V and 1 mM calcium chloride in PBS and subjected to flow cytometry, following the method of De Jong et al. (*13*).

RESULTS

We have examined the effects of disrupting the link among spectrin, ankyrin, and band 3 close to the proximal ends of the dimers, which unite to form tetramers. We have established that the covalent and noncovalent perturbations we have employed to cause this resulted in substantial dissociation of tetramers but had no effect on the interactions of the constituent spectrin dimers at their distal ends with the proteins that define the nodes of the intact membrane skeletal lattice.

Covalent Modification. Intact red cells incubated with NEM under the conditions defined by Smith and Palek (*4*) showed some shape disturbances, with loss of the smooth contour, but no

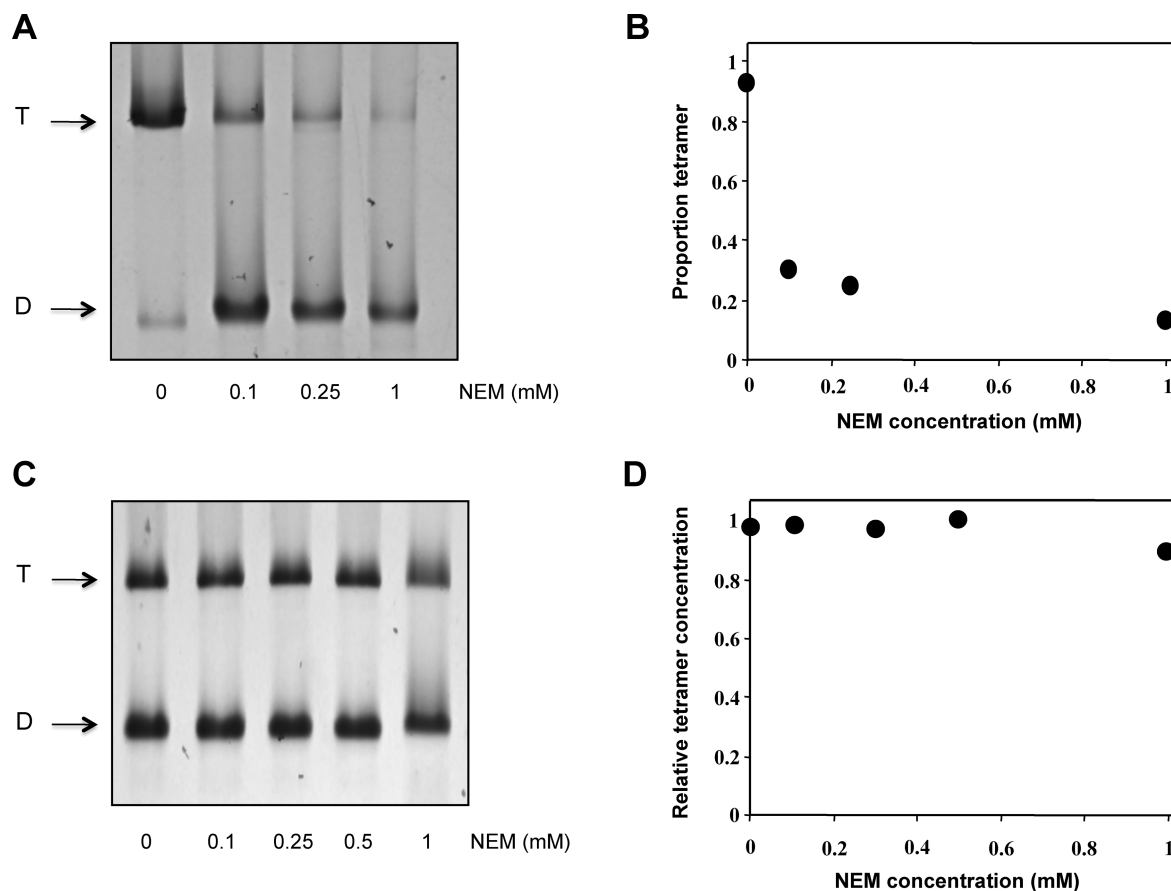**A**

B

C

D

FIGURE 1: Effect of *N*-ethylmaleimide on the association state of spectrin in the red cell membrane and in solution. (A) Native polyacrylamide gel showing separation of the spectrin dimer (D) and tetramer (T), after extraction from cells treated with the indicated concentrations of NEM. (B) Proportion of spectrin converted to dimer in situ, determined by densitometry of the gels. (C) Polyacrylamide gel and (D) densitometric evaluation of the equilibrium mixture of the spectrin dimer and tetramer after treatment in solution with the indicated concentrations of NEM.

appreciable swelling or shrinkage. The level of deformation under shear in the ektacytometer was markedly reduced relative to that of untreated cells, as previously reported (*1*). As observed by Smith and Palek, the fraction of spectrin extracted from the membrane at 4 °C and a low ionic strength in the form of dimers reached a maximum of 70−75%, as revealed by polyacrylamide gel electrophoresis (Figure 1A,B). We examined whether this reflects an inability of this fraction, but not the remaining 25−30% of the dimers, to form tetramers in the NEM-treated cells. We recovered the total spectrin in the form of dimers from the membranes of cells exposed to increasing concentrations of NEM (up to 1 mM) and from untreated control cells by extraction in low-ionic strength medium at 37 °C. The solution was brought to physiological ionic strength by addition of 10-fold-concentrated PBS, and aliquots were incubated at 30 °C for 4 h and analyzed by gel electrophoresis in the cold (Figure 1C,D). The association constant determined from the dimer−tetramer ratios was almost indistinguishable from that of native spectrin under the same conditions, $1.1-1.5 \times 10^6 \, \text{M}^{-1}$ (*9, 14, 15*). Treatment of cells with higher concentrations of NEM gave some evidence of accretion of irreversible dissociation of tetramers. This observation was not pursued further.

To establish whether the self-association sites on the spectrin dimers generated in the cell by NEM treatment were indeed functional in situ, we introduced peptides, corresponding to these sites (*7*), into ghosts derived from the treated cells. These peptides bind competitively to the self-association sites, thereby causing dissociation of the tetramers in situ and in solution (*7*). The peptide derived from αII spectrin (the isoform found in neural cells and many other cells) binds much more strongly and more rapidly than αI or βI (erythroid) peptides to the termini of the erythroid spectrin chains (*8*). The proportions of spectrin dimers and tetramers on the membranes and the amounts of peptide bound were determined by gel electrophoresis as described above. Equilibrium and kinetic data for the binding of peptides to the membranes are shown in Figure 2. The titer of αII peptide bound corresponds, within error, to the full complement of spectrin in both unreacted and NEM-modified membranes (Figure 2A,B). The weaker binding of the βI peptide (Figure 2C) discriminates between the highly reactive free chain termini of the preformed dimers in the NEM-treated cells and the remainder, shielded in the residual tetramers. The apparent association constants for these two populations of sites, calculated from the binding profiles, are $(1.3 \pm 0.1) \times 10^4$ and $(1.5 \pm 0.2) \times 10^3 \, \text{M}^{-1}$, respectively.

It is thus evident that the dissociation by NEM of spectrin into its constituent dimers on the membrane is not caused by modification of the self-association site on either the α- or β-chain. It is in fact only the terminal part of the β-chain that contains cysteine residues (two) [reaction of NEM with amino groups, which is a known though rare occurrence, could be excluded (*5*)]. Because of the weak self-association of spectrin at physiological temperature (*14, 15*), it can be inferred that the predominance of tetramers on the membrane is due to the proximity of the association sites of the constituent dimers. This is imposed by the tight attachment to the membrane of one of the spectrin dimers of each tetramer at a point near its proximal end, where the ankyrin binds to the β-chain
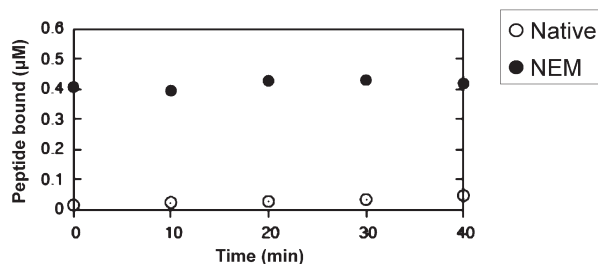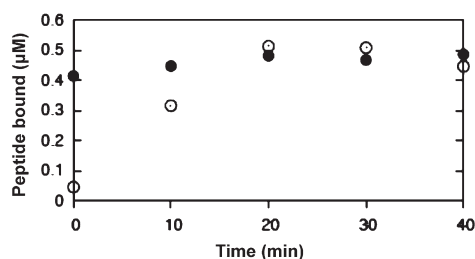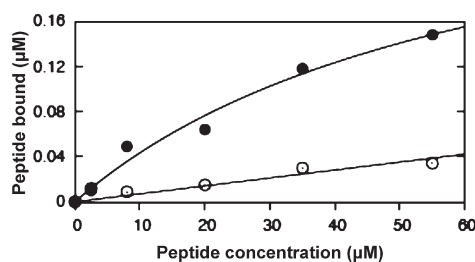
A   Alpha II - 24°C

B   Alpha II - 37°C

C   Beta I - 37°C

FIGURE 2: Equilibria and kinetics of binding of peptides to the self-association sites of spectrin in the red cell membrane. Kinetics of binding to native (○) and NEM-reacted (●) red cell ghosts at 24 (A) and 37 °C (B) of αII spectrin peptide, directed at the spectrin dimer self-association site. (C) Equilibrium binding at 37 °C of βI spectrin peptide, directed at the dimer self-association sites. The curves are best fits, giving apparent association constants (K_{app}) of $(1.3 \pm 0.1) \times 10^4$ and $(1.5 \pm 0.2) \times 10^3$ M^{-1}, respectively, uncorrected for the dimer−tetramer equilibrium (see the text).
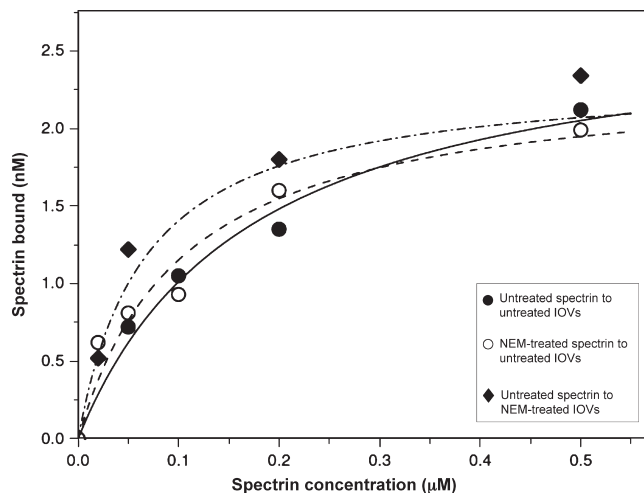

FIGURE 3: Binding of native spectrin to inside-out membrane vesicles from cells treated with *N*-ethylmaleimide and of spectrin from cells thus treated to vesicles of untreated cells. Spectrins bound to vesicles as a function of spectrin concentration: vesicles and spectrin from untreated cells (●). Spectrin from NEM-treated cells and vesicles from untreated cells (○) and spectrin from untreated cells and vesicles from NEM-treated cells (◆). The curves are best fits for binding to a population of identical and independent sites, giving saturation binding titers of 2.8 ± 0.3, 2.6 ± 0.2, and 2.4 ± 0.3 nM, respectively, with corresponding apparent association constants of $(0.6 \pm 0.13) \times 10^6$, $(1.5 \pm 0.3) \times 10^6$, and $(1.0 \pm 0.3) \times 10^6$ M^{-1}, respectively.

lipids, phosphatidylserine (*20−22*) and phosphatidylethanolamine (*23, 24*), ought also to be considered, in view of the evidence that such interactions contribute to the stability of the membrane (*25*), and because NEM is known to inactivate the translocase, partly responsible for maintaining the asymmetric distribution of these lipids (*26*). If, for whichever of these reasons, the proximal ends of the spectrin dimers detach from the membrane surface and thereby acquire an additional degree of freedom of motion, the effective local two-dimensional concentration of association sites would diminish. Weakened association between the dimers would then be expected to ensue.

We have eliminated loss of spectrin−lipid interactions as a factor in the dissociation of tetramers by assaying for exposed phosphatidylserine at the outer cell surface before and after NEM treatment. Flow cytometry of cells labeled with fluorescent annexin V (*13*) revealed no significant difference (data not shown). To determine whether NEM treatment had impaired the spectrin−ankyrin interaction or that of ankyrin with band 3, we examined the binding of spectrin dimers to inside-out vesicles prepared by extraction of spectrin from the membranes of NEM-modified cells and of spectrin from such cells to vesicles from untreated cells (Figure 3). In both cases, binding was essentially unchanged from that of native spectrin dimers to inside-out vesicles from untreated cells.

We last explored the alternative explanation that attachment of spectrin to the membrane is weakened by dissociation of the ankyrin−band 3 bridge. Figure 4 shows the extraction of ankyrin from spectrin-depleted IOVs as a function of salt concentration. Whereas we saw no perceptible extraction from untreated cells at ionic strengths of up to at least 0.25 M, approximately half of their complement of ankyrin was lost from NEM-treated cells during the low-salt extraction of spectrin. At the extraction volume in our experiments, nearly all the remainder was liberated at physiological ionic strength. Similarly, band 3 was much more

in making a bridge to band 3. One way in which the apposition of the dimer−dimer binding sites could be disturbed would be by dissociation of the distal ends of the tetramers from the network junctions, since only one of the dimers in each tetramer is attached to the membrane through an ankyrin bridge (*16*). Such an effect would reveal itself by the disintegration of the isolated skeletal network on treatment with NEM. Membrane skeletons prepared from cells by extraction with Triton X-100 (*17*) showed no loss of spectrin or discernible loss of any of the junction proteins when examined by SDS gel electrophoresis (data not shown). There is thus no disruption of the lattice junctions by NEM, and the spectrin tetramers evidently re-form when the membrane skeletons are liberated [in a greatly condensed state (*18, 19*)] from the lipid bilayer.

This leaves as the likeliest explanation for the dissociation of the spectrin tetramers in the NEM-treated cells the loss of attachments of spectrin to the membrane near the dimer self-association sites. This could result from breakage of either the spectrin−ankyrin or ankyrin−band 3 link, since the ankyrin-binding site in spectrin is located on the β-chain, near its C-terminus, where it binds to the α-chain of the partner dimer. Binding of spectrin to inner leaflet

A

Western Blot anti-Ankyrin

- NEM

+ NEM

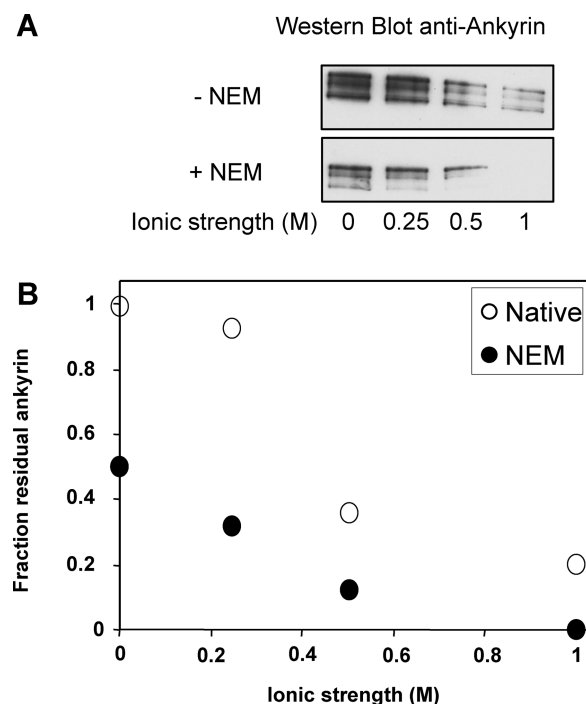Ionic strength (M)   0   0.25   0.5   1

B

FIGURE 4: Extraction of ankyrin from inside-out vesicles of cells before and after treatment with *N*-ethylmaleimide as a function of the ionic strength of the extracting medium. (A) Residual ankyrin on the membranes as a function of ionic strength of the extraction buffer in spectrin-depleted inside-out vesicles before and after NEM treatment as documented by Western blot analysis. (B) Fractions of ankyrin remaining in the vesicles after extraction: NEM-treated cells (●) and control cells (○). Note that in the former, the proportion of residual protein refers to the ankyrin present in the vesicles as prepared, which have already lost nearly half their ankyrin during the course of preparation.

readily extracted by detergent-containing solutions (*17*) from the membrane skeletons of NEM-treated than untreated cells (data not shown). Ankyrin, accordingly, is barely retained in the membranes of the modified cells. We conclude that NEM does indeed sever the attachment of spectrin to the membrane by eliminating or greatly weakening the ankyrin−band 3 interaction.

Noncovalent Modification. To determine whether the spectrin−membrane link can also be disrupted without chemical modification, we prepared a spectrin β-chain fragment, βIR-(14−15), containing the ankyrin-binding site (*27*). Introduction of increasing concentrations (up to 50 μM) of this peptide into ghosts led to a substantial loss of stability of the membrane under shear in the ektacytometer (Figure 5A). Moreover, spectrin extracted from the membrane by dialysis in the cold revealed the presence of 15−20% dimers (Figure 5B). We are unable to determine what proportion of the spectrin−ankyrin links were dissociated under these conditions (the maximum attainable peptide concentration), but it is clear that, as in the NEM-treated cells, the partial dissociation of the spectrin from its primary site of attachment to the membrane is accompanied by partial dissociation of the spectrin tetramers.

DISCUSSION

Our results raise some questions regarding the generally accepted schemes, elaborated over the past several decades, to explain the remarkable elastic properties and the exceptional resistance to mechanical stress of the red cell membrane. Our observations confirm that the predominant self-association of
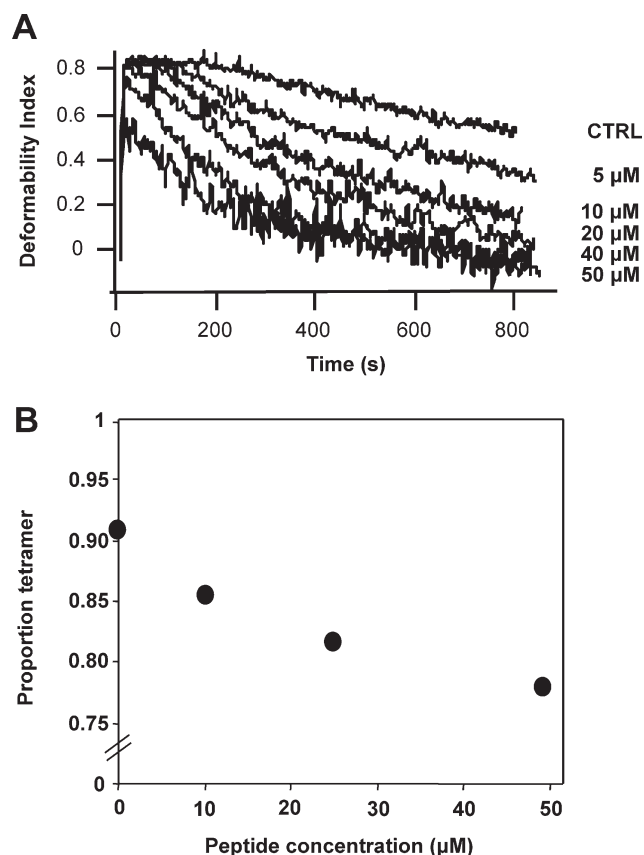
A

B

FIGURE 5: Effect of incorporation of the β-spectrin peptide that binds to ankyrin [βIR(14−15)] on membrane mechanical stability and spectrin dimer−tetramer equilibrium. (A) Membrane mechanical stability of the resealed ghosts was measured by ektacytometry. Membrane stability, expressed as the rate of decline of the deformability index, DI, diminishes (decay curve displaced toward shorter times) with increasing concentrations of peptide incorporated into cells. (B) Densitometric evaluation of native electrophoretic gels of spectrin extracted from cells treated with peptide, showing partial dissociation of spectrin tetramers.

spectrin dimers at physiological temperature in situ, despite their weak intrinsic affinity for one another in free solution, depends on their close apposition on the membrane. This depends in turn on the band 3−ankyrin bridge to the lipid bilayer. Our model for the relation between the disruption of spectrin−membrane association and spectrin tetramer dissociation is schematically depicted in Figure 6.

Our finding that partial dissociation of spectrin tetramers can be induced by a noncovalent disturbance of the spectrin−membrane nexus is consistent with the similar effects exerted by genetic anomalies. Thus, Agre et al. (*28*) found a large deficit of ankyrin-binding sites on spectrin- and ankyrin-denuded inside-out vesicles, isolated from cells of patients with elliptocytic hemolytic anemia, characterized by the presence of spectrin dimers. Another spectrin variant with defective self-association, leading to severe hemolytic anemia, was found to engender impaired ankyrin binding (*29, 30*). The consequence of rupture of the spectrin−ankyrin−band 3 complex may be somewhat mitigated by the interaction between spectrin and the phospholipids of the inner membrane leaflet (*25*), but this is clearly insufficient to compensate for the suppression of the spectrin−ankyrin interaction by the ankyrin-binding spectrin peptide. At the same time, the attachment of ankyrin to spectrin dimers has been found to cause a 10-fold increase in the self-association equilibrium constant (*31*). The reaction with NEM
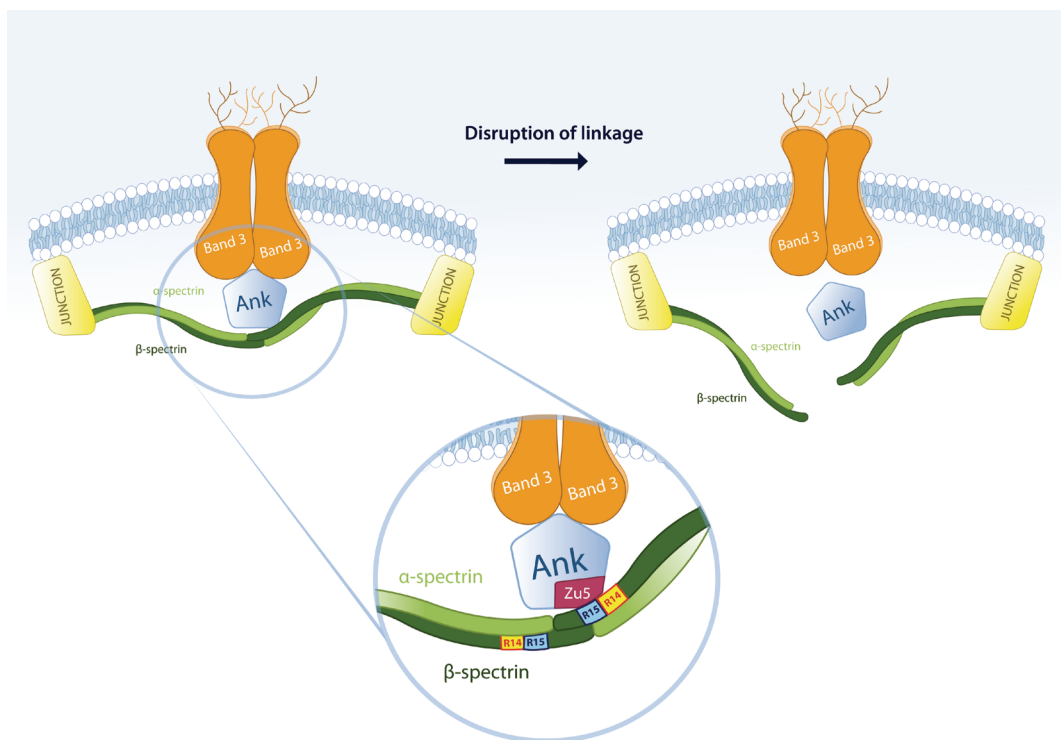
FIGURE 6: Schematic model showing suggested consequences of disruption of the spectrin−ankyrin−band 3 bridge on spectrin self-association. The spectrin α-chains are colored light green and the β-chains dark green. In the inset, the interactions between ankyrin and β-spectrin are highlighted. Zu5, a subdomain of ankyrin, binds to β-spectrin through the terminal part of R14 and most of R15 and has been identified as the minimal binding domain for β-spectrin (*27, 39*).

leaves the ankyrin−spectrin association undisturbed, and therefore, this consideration does not presumably enter into the NEM-induced dissociation of the spectrin tetramers. We cannot, however, exclude its participation in the noncovalent intervention.

We have established that NEM causes dissociation of spectrin tetramers without perturbing the dimer self-association sites, that the spectrin−ankyrin link is unaffected, and that the site of disruption of the spectrin−membrane interaction lies at the ankyrin−band 3 interface. The results explain the observation (*4*) that NEM causes dissociation of no more than ∼75% of the spectrin tetramers (and higher-order oligomers) into dimers in the native membrane. We cannot determine whether the capacity of band 3 to bind to ankyrin is eliminated or merely weakened, for there is evidence that band 3 contains two binding sites for ankyrin (*32*) (which may account for the tetrameric state of the ankyrin-bound band 3, while the remainder is dimeric). In addition, the extraction of band 3 from the preparations of membrane skeletons from NEM-treated cells is incomplete at physiological ionic strength; this implies that an interaction with some membrane component survives NEM treatment. Proteins known to interact directly or indirectly with band 3 include 4.1R (*33*) and adducin (*34*) and, among transmembrane constituents, glycophorin A and a number of blood group proteins (*35*).

We also established that the spectrin β-chain peptide, which contains the binding site for the α-chain, binds much more avidly to NEM-treated cells than to native cells. This bears out the conclusion that dimer self-association sites become available on treatment of the cell with NEM. Analysis of the interaction of the peptide with the spectrin in the NEM-treated cells now permits an estimate of the intrinsic affinity of the univalent peptide for free dimer ends on the membrane, and therefore of the strength of the dimer−dimer interaction in the unperturbed cell (*7*). Binding of the peptide to the native membrane (Figure 2C) is governed by an apparent association constant. $K_{app} = [PS]/[P][S]$, where [PS] represents the concentration of bound peptide, [P] that of free peptide, and [S] that of free binding sites; since in these experiments the total peptide concentration $P_o \gg [PS]$, $K_{app} \sim [PS]/P_o[S]$. However, the peptide binds only to free α-chain ends, and thus, the true association constant is $K = [PS]/P_o[O]$, where [O] represents the concentration of "open" dimers (twice the number of dissociated tetramers). Here $[O] = S_o − [PS] − [C]$, where S_o is the total concentration of membrane-associated spectrin in terms of dimers and [C] that of self-associated ("closed") dimers. The dimer−tetramer balance can be expressed in terms of a (dimensionless) pseudoequilibrium constant $K' = [O]/[C]$. In the NEM-treated cells, with ca. 75% dimers at equilibrium, $K'_{NEM} \sim 3$, whereby the true association constant, K, between a univalent β-chain and the α-chain of an open dimer in situ can be estimated. This emerges as ∼1.7×10^4 M^{-1} (Figure 2B). In the native, unreacted cell, the open−closed equilibrium is defined by a constant K'_o, analogous to K'_{NEM}. Binding of the peptide to the unreacted membrane yields an apparent association constant, $K°_{app}$, of ca. 1.3×10^3 M^{-1} (Figure 2C). From this, given the value of K, we can make an estimate of K'_o, the ratio of free to self-associated dimers on the unperturbed membrane. K'_o, as here determined, refers to binding of a univalent peptide to a divalent dimer; however, in solution, the association constants between two univalent α- and β-fragments and between two divalent αβ-fragments differ by no more than a factor of ∼2 (*14*), and we may reasonably assume that a similar relation prevails on the membrane. This leads to an estimate of 0.08 for K'_o, or in other words, some 8% of the dimers in the unperturbed membrane are in the dissociated (open) state. This conforms to the small proportion of spectrin dimers, which appears always to be extracted from the membrane at low temperatures, when the dimer−tetramer equilibrium is effectively frozen (*14, 15*). We stress that, given

the experimental error, considerations of temperature and solvent conditions, possible molecular crowding by hemoglobin, uncertainty about whether the dissociated components of a dimer can both bind a peptide molecule, and other assumptions implicit in the calculation, the accuracy of the estimate of K'_o is limited. Nevertheless, the implication stands that the association of the dimers on the membrane is quite weak (though of course much stronger than in solution).

The dependence of the ability of the membrane to resist shear stresses on the maintenance of spectrin in its tetrameric state is well-established and -understood (*1, 3*). Why an increase in shear modulus should accompany the dissociation of a large proportion of the elastomeric members of the membrane skeletal network, as seen in NEM-treated cells and cells of HE patients with membranes containing a large proportion of spectrin dimers (*36*), remains unexplained. The increased degree of freedom of the pendant spectrin dimers could perhaps allow them to form interactions with other proteins, and so impede the deformation of the membrane, or their association with phospholipids of the inner membrane leaflet could become similarly favored. Three structural perturbations by which the cell can adapt to shear-induced deformations have been recognized: (1) the spectrin tetramers can increase, by perhaps 3-fold, their end-to-end separation, which in the unperturbed cell is constrained well below its equilibrium value (see, for example, ref *19*); (2) the spectrin tetramers can dissociate transiently into dimers (*7, 8*); (3) some of the repeating units, of which the spectrin chains are composed, can unfold (*3, 37*). In the case in which the spectrin dimer−tetramer equilibrium strongly favors the dimer, all these responses would presumably be restricted to the minority of spectrin molecules in the tetrameric state at any instant. The dissociation of the spectrin tetramers leads to relatively unimpeded translational diffusion of transmembrane proteins (*38*) and may therefore be expected to allow them to cluster, and possibly also bind to membrane skeletal constituents. Such interactions could conceivably render the membrane more rigid. A definitive explanation of the relation between the continuity of the membrane skeletal network and the elastic characteristics of the membrane demands more extensive investigation.

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