Elasticity of the Red Cell Membrane and Its Relation to Hemolytic Disorders: An Optical Tweezers Study

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ABSTRACT We have used optical tweezers to study the elasticity of red cell membranes; force was applied to a bead attached to a permeabilized spherical ghost and the force-extension relation was obtained from the response of a second bead bound at a diametrically opposite position. Interruption of the skeletal network by dissociation of spectrin tetramers or extraction of the actin junctions engendered a fourfold reduction in stiffness at low applied force, but only a twofold change at larger extensions. Proteolytic scission of the ankyrin, which links the membrane skeleton to the integral membrane protein, band 3, induced a similar effect. The modified, unlike the native membranes, showed plastic relaxation under a prolonged stretch. Flaccid giant liposomes showed no measurable elasticity. Our observations indicate that the elastic character is at least as much a consequence of the attachment of spectrin as of a continuous membrane-bound network, and they offer a rationale for formation of elliptocytes in genetic conditions associated with membrane-skeletal perturbations. The theory of Parker and Winlove for elastic deformation of axisymmetric shells (accompanying paper) allows us to determine the function BH^2 for the spherical saponin-permeabilized ghost membranes (where B is the bending modulus and H the shear modulus); taking the literature value of 2×10^{-19} Nm for B, H then emerges as 2×10^{-6} Nm^{-1}. This is an order of magnitude higher than the value reported for intact cells from micropipette aspiration. Reasons for the difference are discussed.

INTRODUCTION

The red blood cell membrane has unique viscoelastic properties, which resemble, in some respects, those of a fluid, in others, those of a solid. Their structural basis remains a matter of debate. The high resistance of the membrane to changes in surface area is a characteristic of phospholipid bilayers, whereas its response to shear deformation depends on the cytoskeletal network of proteins that coats its cytoplasmic surface (Evans and Hochmuth, 1978; Berk et al., 1989; Mohandas and Evans, 1994).

The distortion of the cell in response to an applied mechanical stress has been observed in a variety of ways, but nearly all quantitative data on elastic and rheoviscous properties are the outcome of one technique. This is micropipette aspiration (Evans, 1973), in which a protrusion from a flaccid membrane is created and drawn into the pipette. The relation between pressure and extension of the protrusion then delivers a value of the shear elastic modulus. The relaxation rate, when the pressure is released, can also give an estimate of the shear viscosity.

We have explored the scope of the optical tweezers technique for applying a defined linear stretching force to the red cell membrane and measuring the response to fast and slow induced distortions. We have sought to define the structural features of the membrane skeleton that control the elastic properties and to relate them to the effects of hereditary cytoskeletal anomalies. The results show several unexpected features and differences from earlier data.

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MATERIALS AND METHODS

Preparative and analytical procedures

Red blood cells were obtained from the blood bank and were no more than a week old. To prepare ghosts, cells were three times washed with isotonic phosphate-buffered saline (PBS), pH 7.6, the upper layer, containing leukocytes, being discarded each time. The cells were then suspended at 10% hematocrit and 0.1 volume of 10 mg ml^{-1} saponin was added, together, in some cases, with 1 μl/ml of cell suspension of 20 mg ml^{-1} phenylmethylsulphonyl fluoride in ethanol. The cell suspension was left at room temperature for 20 min and the ghosts and residual unlysed cells were then pelleted at 40,000 $\times$ g; the pale ghost layer was collected and washed three more times with PBS. Ghosts obtained by hypotonic lysis were also examined. These were prepared by adding the packed, washed cells to 20 volumes of ice-cold 10 mM sodium phosphate, pH 7.6, together with phenylmethylsulphonyl fluoride. Ghosts were collected by pelleting as before and washed 2–3 times with the lysis medium. They were then made isotonic in PBS and 1 mM in magnesium-ATP, by addition of a ten-times concentrated stock solution, and incubated for 1 h at 37°C.

To determine whether saponin lysis had caused loss of cholesterol, total lipid was extracted with 2:1 chloroform-methanol from membranes prepared by hypotonic and saponin lysis. Phospholipid in the extract was assayed by ashing with perchloric acid and determining the orthophosphate concentration by the ammonium molybdate color reaction, whereas cholesterol was assayed by the ferric chloride color reaction. Analytical procedures were as set out by Kates (1972).

To examine how the mechanical properties of the membrane are related to the known protein–protein interactions that maintain the integrity of the membrane-associated network, three types of modification were undertaken. The targets of the three modifying agents are depicted schematically in Fig. 1 *A* and the results of the modifications, as revealed by gel electrophoresis, are shown in Fig. 1 *B*.

To effect dissociation of the spectrin tetramers to dimers in situ (Fischer et al., 1978), the saponin-lysed ghosts were suspended at their original concentration in PBS, adjusted to pH 7.0. The suspension was then made 2 mM in *N*-ethylmaleimide (NEM) and left to react at room temperature for

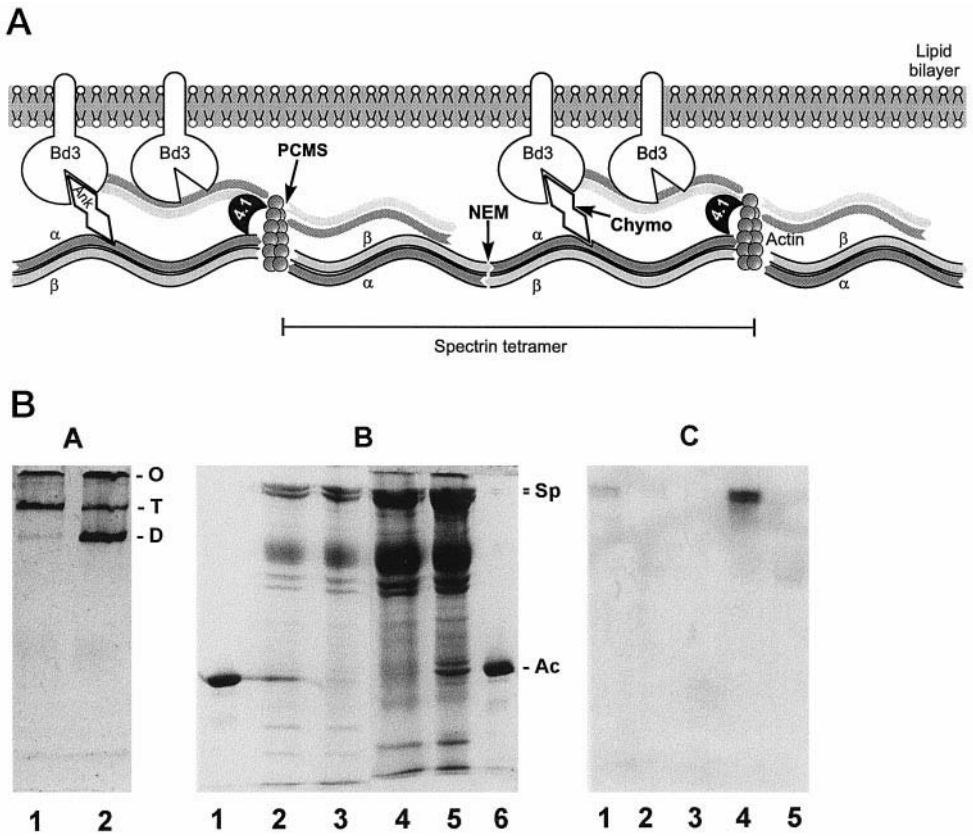

FIGURE 1 (*A*) Schematic representation of the red cell membrane structure and sites of functional modifications. The membrane skeletal network is composed of an approximately hexagonal lattice, in which the structural members are spectrin tetramers, made up of $\alpha\beta$ heterodimers, associated head-to-head. The junctions of the lattice consist of short actin filaments, together with protein 4.1, which binds to spectrin and actin. Each spectrin tetramer is attached by way of an ankyrin molecule to a population of the transmembrane protein, band 3. Arrows show the sites of action of the modifying reagents. NEM dissociates some 70% of the spectrin tetramers to dimers, PCMS causes dissociation of the actin protofilaments, with complete loss of actin from the membrane, and chymotrypsin (Chymo) severs the ankyrin, and thus the link between spectrin and band 3, without significantly degrading other proteins. (*B*) Modifications to membrane structure. *Panel A*, Dissociation of spectrin tetramers in situ by treatment with NEM: spectrin was extracted in the cold from NEM-treated ghosts. Gel electrophoresis shows that spectrin from untreated ghosts (*lane 1*) remained almost entirely tetrameric, whereas that from the modified ghosts was largely (~70%) dissociated into dimers (*lane 2*). O denotes the origin of migration (top of the gel). The staining here represents the oligomeric fraction of spectrin, associated with actin and 4.1. T is spectrin tetramer, and D the dimer. *Panel B*, Dissociation of membrane skeletal actin protofilaments by treatment of ghosts with *p*-chloromercuriphenyl sulphonate. SDS-polyacrylamide gel electrophoresis shows membrane proteins of untreated ghosts applied at two concentrations (*lanes 2 and 5*) and of treated ghosts at the same concentrations as the untreated (*lanes 3 and 4*). Lanes 1 and 6 show the migration of pure actin. Sp denotes the spectrin doublet and Ac actin. The only proteins that can be seen to have been affected by the reagent are actin and the minor actin-binding protein, band 4.9, migrating just above the actin. *Panel C*, Proteolytic cleavage of ankyrin with chymotrypsin: electroblot of SDS-gel, stained with anti-ankyrin followed by second antibody. The undigested ghosts were applied on lane 4 and purified spectrin on lane 3. Lane 1 shows traces of ankyrin remaining after a digestion of 6 min, whereas no ankyrin is detected after a digestion of 180 min (*lane 2*) or after 20 min (*lane 5*), the conditions used to generate the modified ghosts for the optical tweezers experiments. The gel stained with Coomassie Brilliant Blue R revealed no detectable loss of any other zones after the 20-minute treatment.

1 hr. The ghosts were recovered by pelleting, a twofold molar excess of dithiothreitol over the original NEM concentration was added, and the ghosts were washed twice with PBS, pH 7.6. To assay the extent of tetramer dissociation, the spectrin was extracted by washing the ghosts with ice-cold 0.25 mM phosphate, pH 8, and dialyzing against the same buffer in the cold. The spectrin was recovered in the supernatant after centrifugation at 100,000 $\times$ *g* for 15 min and analyzed by electrophoresis in a 4.5% polyacrylamide gel in a 0.1 M Tris-Bicine buffer system, run in the cold. The gel, stained with Coomassie Brilliant Blue R250, was evaluated by densitometry (Fig. 1 *B*, *panel A*). The spectrin tetramers were also dissociated in situ by incubating ghosts in 10 mM sodium phosphate, pH 8.0, at 37°C for 1 hr (Liu and Palek, 1980). They were then made isotonic and kept cold. The spectrin was extracted and analyzed by gel electrophoresis in the cold, as above.

Membrane-skeletal actin was dissociated from the protein network by the method of Gordon and Ralston (1990): ghosts were washed with 5 mM phosphate, pH 8.2, and pelletted. To the loose pellet 0.1 volume of 10 mM *p*-chloromercuriphenylsulphonic acid (PCMS), adjusted to pH 8.0, was added, and the reaction was allowed to proceed for 1 h on ice. The pellet was then suspended in PBS, pH 7.6, containing a fivefold molar excess of dithiothreitol over PCMS, and the ghosts were washed twice with PBS. To determine the extent of extraction of the actin, an aliquot of the ghost preparation was dissolved in 1% sodium dodecyl sulphate, diluted tenfold with water, followed by 0.1 volume of a ten-times concentrated polyacrylamide gel sample buffer (Laemmli, 1970), containing sucrose, β-mercaptoethanol, and Bromophenol Blue tracker dye, heated at 100°C for 5 min and applied to a 9% sodium dodecylsulphate-polyacrylamide gel. Muscle actin was also applied to the gel to ensure correct identification of the actin

zone (Fig. 1 *B*, *panel B*). To determine whether the reagent had caused dissociation of spectrin tetramers, spectrin was extracted in the cold, as described above, from an aliquot of the treated ghosts and analyzed by polyacrylamide gel electrophoresis in the cold in the absence of denaturant.

Cleavage of ankyrin in unsealed hypotonic ghosts occurs on very mild treatment with trypsin (Jinbu et al., 1984) or chymotrypsin (Pinder et al., 1995), with little or no detectable damage to other proteins. We found that scission of ankyrin in ghosts derived from saponin-lysed cells at physiological ionic strength required considerably greater exposure to the enzyme, but that the other membrane-associated proteins were correspondingly more resistant. Freshly dissolved chymotrypsin was added to ghost pellets on ice to give a concentration of 10 μg ml^{-1}. Aliquots taken at various times were quenched with excess chymostatin. The ghosts were washed twice with PBS and prepared as before for SDS-polyacrylamide gel electrophoresis. To analyze the residual content of intact ankyrin the ghost protein was dissolved in SDS-gel sample buffer as before and separated in 5.6% gels in the buffer system of Fairbanks et al. (1971). Duplicate gel lanes were stained with Coomassie Brilliant Blue R250 or electroblotted onto nitrocellulose membrane. The membrane was blocked with milk powder, then treated with a rabbit polyclonal anti-ankyrin antibody (prepared by J.C. Pinder in this laboratory, Randall Institute, King's College, London) and the blots were developed with second antibody, using the chemiluminescent (ECL) system (Amersham, Little Chalfont, U.K.). Analysis by this method revealed that, under the above conditions, a chymotryptic digestion of 20 min sufficed to eliminate all intact ankyrin, band 2.1 (Fig. 1 *B*, *panel C*). Electrophoresis revealed no detectable damage to any other proteins; in particular, protein 4.1, which is very sensitive to proteolysis, remained intact. In addition, we extracted spectrin from the chymotrypsin-treated ghosts and examined it electrophoretically as above, to show that there was no conversion of tetramers to dimers.

Giant phospholipid vesicles with the composition of the red cell membrane, prepared by the method of Käs and Sackmann (1991), were given to us by Dr. P. McCauley (Imperial College, London).

Polystyrene latex beads of 1 μm diameter, derivatized with aldehyde groups (Interfacial Dynamics Corp., Portland, Orgeon), were incubated for 2 h at room temperature with 0.1 mg ml^{-1} wheat germ agglutinin (Sigma, Poole, U.K.) in 0.3 M sodium chloride, 40 mM sodium borate, pH 8.2. An excess of neutralized glycine was then added, and the beads were washed by pelleting from suspensions in PBS. For experiments on the phospholipid vesicles, the beads were similarly reacted with annexin V (Alexis Corp., San Diego, California), which had first been dialyzed against the reaction medium to remove the admixture of glycine added by the manufacturer.

Optical tweezers

The apparatus was based on an inverted microscope (Zeiss Axiovert, Oberkochen, Germany). Polarizing prisms were used to split the laser beam (Nd-YLF 1.047 μm TFR, Spectra Physics, Mountain View, California) to give two independently movable single-beam gradient traps, the stiffnesses of the two traps being equalized with a $\lambda/2$ plate. A 63×, infinity-corrected, 1.4 N.A. objective was used for these studies. Trap stiffness was measured from the Brownian motion of a trapped bead, and the values derived from the corner frequency and from the standard deviation of the bead position were in satisfactory agreement (0.08 pN nm^{-1}). In the experiments, one of the traps was left stationary, and the position of the bead in this trap was monitored by focussing an image onto a photodiode quadrant detector. The position of the second bead was controlled by a pair of acousto-optical modulators, which allowed the trap to be moved rapidly over a range of about 4 μm. The method measured the compliance of the link between the two beads but did not exclude the possibility that the two ends of the cell, or more likely the bead cell linkages at each end, were behaving differently. To address this question, the positions of the two beads relative to the center of the cell were analyzed in a series of 25 video frames. On average, the compliance of the link between each of the two beads and the center of the cell differed by 30%. The compliance of the stronger of the two links, which might be regarded as the best estimate, is about 15% less than the average value we report.

RESULTS

Principles of the measurements

The principle of the optical-tweezers approach to the measurement of elastic properties consists in attaching two adhesive beads to the cell at opposite ends of a diameter, and holding one in place with one trap, while moving the other with a second trap, to induce a tension (positive or negative) in the cell. By monitoring the movement of the first bead in response to the controlled displacement of the second bead, a force-extension profile can be generated. Two types of force-extension curves were investigated: in the first, the periodic method, a triangular wave motion (1–2 Hz, which is well below the frequency at which a lag in response, indicative of viscous relaxation, develops) was imposed on the moving trap; in the second, the stepwise method, a series of stepped increases in length were imposed on the cell, the time between steps being such as to allow the tension in the cell to relax back to close to its equilibrium value and thus reveal whether it undergoes plastic yield.

A number of procedures were tried for generating beads that would attach tightly to the cell exterior. The best results were obtained with aldehyde-derivatized beads, to which wheat germ agglutinin was covalently coupled. This lectin has high affinity for the carbohydrate of the exposed sialoglycoproteins, the glycophorins. Beads prepared in this way were used for all studies described here, other than those on synthetic phospholipid vesicles.

Properties of red cell ghosts

The application of the optical-tweezers technique to the native biconcave cell or to the resealed ghost presents a number of problems. The opacity of intact cells reduces the precision with which the position of an attached bead can be determined with the quadrant detector. More fundamental is the difficulty of interpreting force-extension profiles for objects that do not have rotational symmetry about the axis joining the beads. Finally, because of the impermeability of the membrane, intact cells change shape under conditions of constant volume, which means that the response to strain is very dependent upon the pressure difference across the membrane; the mechanical properties of the protein cytoskeleton may then no longer be the dominant influence on the force-extension curve. For these reasons, we have chosen to study ghosts prepared by saponin lysis. Saponin belongs to a group of glycosylated sterols, which includes digitonin, and interacts specifically with membrane cholesterol to cause permeabilization of the membrane (Elias et al., 1978). The holes are too small to be visible in the electron microscope, although they allow the passage of large proteins (Seeman, 1967). The only perceptible structural changes in the membrane are the appearance of small surface pits, 40–50 Å across (Seeman et al., 1973), and corrugations in the freeze-fracture faces (Elias et al., 1978).

We have found that ghosts generated in this manner are mainly spherical, with a smooth contour and a relatively uniform appearance. The membrane composition appears to be unchanged, or at all events, the cholesterol:phospholipid ratio was the same as that in ghosts prepared by hypotonic lysis within the precision (~5%) of our assay. Saponin-lysed cells were previously shown to freely admit proteins, such as G-actin and gelsolin (Pinder et al., 1986). We have further shown that, when spectrin, labeled with fluorescein isothiocyanate (freed of excess reagent by gel filtration), was added to the ghosts, the fluorescent intensity inside the ghost had become indistinguishable from that in the surrounding buffer within the time (a few seconds) between mixing and observation. Resealed hypotonically generated ghosts, by contrast, showed no penetration of fluorescence into the lumen. Considering the difference of two orders of magnitude between the diffusion coefficients of spectrin and of water, we therefore take it that volume equilibration in the saponin-lysed ghosts is effectively instantaneous, and the constant-volume restriction is therefore eliminated. The uniformity of shape is an additional important advantage for optical trap experiments.

Comparison of the saponin-treated ghosts with intact red cells and with smooth resealed hypotonically generated ghosts shows that the force-extension curves are broadly similar, and it is therefore likely that the elastic characteristics of the membrane are little changed by exposure to saponin.

On application of the maximum force used in this study of 25 pN, the axial ratio of the ghost increased from unity to about 1.2. The cells were also compressed for half the cycle, but, in this case, it cannot be ensured that the pressure remains orthogonal to the membrane surface, and lateral displacement of the bead was often observed. No quantitative analysis in the compressive part of the force-extension profile was therefore attempted.

Phosphorylation of membrane proteins

Incubation of the ghosts with magnesium-ATP to phosphorylate spectrin and other membrane skeletal proteins made no detectable difference to the shape of the ghost or to the force-extension profiles. This is consistent with the lack of any such metabolic effect on the elastic properties of red cells, measured by the micropipette technique (Meiselman et al., 1978) (though, of course, in intact cells or resealed ghosts, ATP depletion induces shape changes). The ATP incubation was therefore omitted in most experiments.

Effects of structural perturbations of the membrane skeleton

Modifications of the membrane cytoskeleton in the ghosts were undertaken in an attempt to identify the structural features associated with the elastic properties. The protein–protein interactions responsible for the cohesion of the membrane-cytoskeletal complex can be separated into "horizontal" and "vertical" kinds, that is, those in the plane of the skeletal network and those orthogonal to the plane of the membrane and affecting, therefore, the membrane–network interaction. Many genetic defects in both these categories have been discovered and are associated with characteristic pathological phenotypes (Lux and Palek, 1995). Figure 1 *A* shows, in schematic form, the sites of the modifications that we have carried out.

The most common horizontal defects, which give rise to hereditary elliptocytosis and hemolytic disease, are spectrin mutations in the self-association site of the $\alpha\beta$-dimers that form the structural members of the network (spectrin tetramers) by interacting head-to-head. This condition can be reproduced by treating the ghosts with *N*-ethylmaleimide (Fischer et al., 1978) or by incubating them at low ionic strength (Liu and Palek, 1980). Both treatments dissociate a maximum of about 70% of the spectrin tetramers into dimers (Fig. 1 *B*, *panel A*). Most of our experiments were performed on the *N*-ethylmaleimide-treated ghosts because of the slow reversal of the electrostatically induced dissociation when the ghosts are returned to an isotonic medium at room temperature.

A still more radical disruption of the membrane skeletal network can be achieved by dissociating the short actin filaments that make up the lattice junctions, with complete loss of actin from the membrane. The thiol-specific organomercurial, PCMS, has been shown to react with membrane skeletal actin, as well as with spectrin, and only to a small extent with any other major membrane-associated proteins (Gordon and Ralston, 1990). We found, in agreement with these workers, that, under their conditions of reaction, the actin was almost entirely lost (Fig. 1 *B*, *panel B*). Extraction of these ghosts at low ionic strength in the cold liberated only a minor proportion of the spectrin, but this fraction was tetrameric. It seems likely, therefore, that the action of the reagent is effectively confined to the elimination of actin, together with the minor actin-binding protein, band 4.9, as observed by Gordon and Ralston (1990).

To examine the effects of disturbing vertical interactions in the membrane, we found conditions for cleaving the ankyrin by proteolysis, with no discernible degradation of any other proteins. These were based on earlier observations on proteolysis of open ghosts at low ionic strength (Jinbu et al., 1984; Pinder et al., 1995). We found that all the ankyrin was degraded (Fig. 1 *B*, *panel C*), presumably into its spectrin- and band 3-binding domains (Hall and Bennett, 1987). The resulting ghosts were somewhat irregular in outline and showed some tendency to throw off vesicles.

Force-extension profiles

The response of an unmodified ghost to a periodic applied force and a typical force-extension relationship are shown in

Fig. 2. Two factors that could be imagined to limit the reproducibility of the measurements are the natural variation in cell size within a single blood sample (Jay, 1975) and the precision with which the two beads can be positioned at opposite ends of a diameter. However, the theory of Parker and Winlove (1999) predicts that the stiffness will be dependent on only the cube-root of the radius, and, over the small range of cell sizes in a population, we found no significant correlation between cell size and stiffness. We also found that, when the beads were deliberately positioned off the diameter, there was only a modest difference in the measured response. Averaged force-extension profile for normal and variously modified red cell ghosts are shown in Fig. 3.

Around the point of zero extension, all the modified cells showed a fourfold diminution in stiffness, relative to the untreated controls, but the difference from normals was strikingly reduced to a factor of only about 2 at the highest applied forces (Fig. 3, *B–D*). The observations on the NEM-treated ghosts stand in contrast to the somewhat elevated shear elastic modulus derived from micropipette aspiration (Chabanel et al., 1989: Rangachari et al., 1989).

Theoretical analysis

The accompanying work (Parker and Winlove, 1999) analyses the response to tension applied at opposite poles of a spherical shell. Resistance to polar extension arises from two sources, the out-of-plane bending stiffness, B, and the in-plane shear modulus, H. Their relative contributions are expressed in terms of the nondimensional parameter $C = a^2H/B$, where a is the radius of the sphere. The analysis shows that, for values of $C > 10$, if the dimensional force F^* is scaled, such that $F_s = F^*(aBH^2)^{-1/3}$, a plot of F_s against the fractional extension, ϵ, is almost independent of C. This leads to the approximation: $F^* \approx 5\epsilon(aBH^2)^{1/3}$, from which BH^2 can be directly determined. For the unmodified ghosts, a fractional extension of 0.1 gives a force of about 15 pN, leading to a value for BH^2 of 9×10^{-27} N^3m^{-1}. The modified ghosts all behave in a fairly similar manner, an extension of 0.1 giving forces of 4, 4.9, and 3.6 pN for the chymotrypsin-, PCMS-, and NEM-treated ghosts, respectively. These correspond to values of 1.7×10^{-28}, 3.14×10^{-28}, and 1.3×10^{-28} N^3m^{-1} for BH^2.

As will be discussed, B can probably be taken to be a function of the lipid bilayer, and should thus be little affected by modifications of the associated proteins. Literature values of B lie in the range $1.8–7 \times 10^{-19}$ Nm (Evans, 1983; Strey et al., 1995). If we assume Evans's preferred value of 2×10^{-19} Nm, then for unmodified cells, H must be about 2×10^{-4} Nm^{-1}, and for modified cells, about 3×10^{-5} Nm^{-1}, corresponding to values for C of 9000 and 1300: the requirement for $C > 10$ is thus met. The protein modifications reduce the force developed for a given small extension by a factor of about 4, but because of its dependence on $H^{2/3}$ the shear modulus is changed by a factor of about 7.

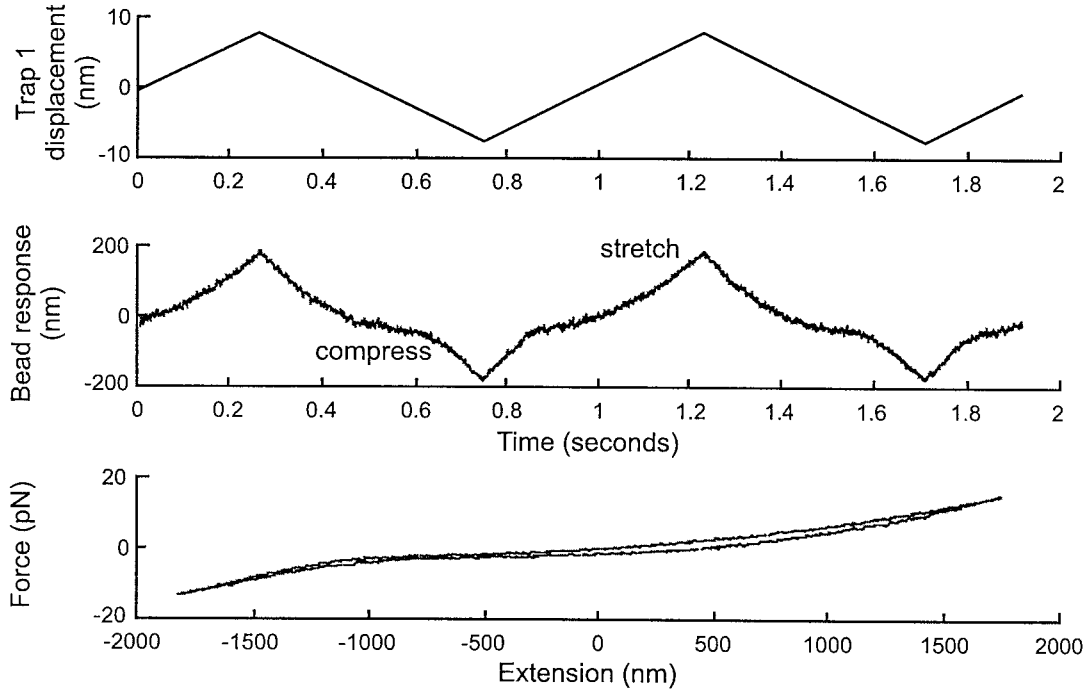

FIGURE 2 (*A*) Movement of the driven bead in response to a periodic tensile force in the form of a triangular wave-form. The response of the indicator bead is shown in *B*, while *C* shows a typical force-extension profile for a normal ghost, the upper curve corresponding to a stretch and the lower to the ensuing relaxation.

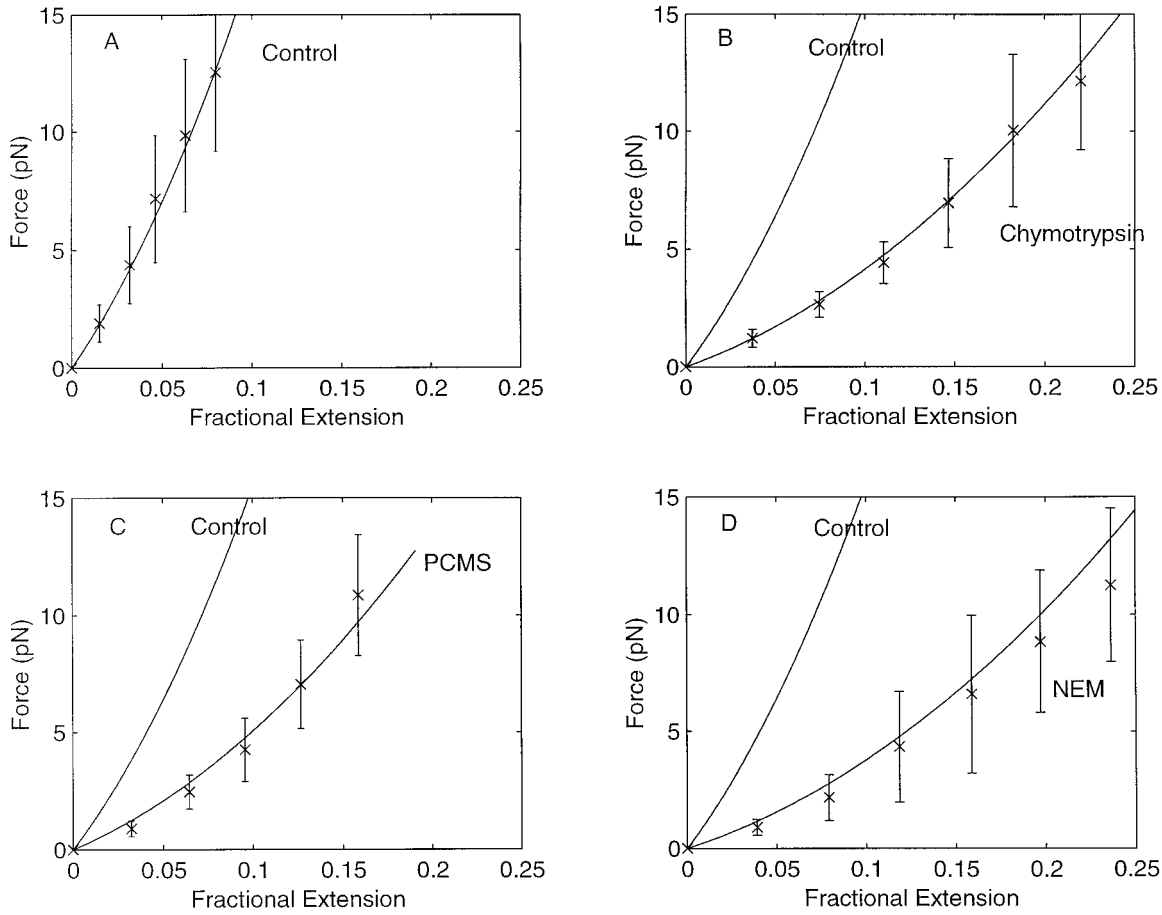

FIGURE 3 The dynamic force-extension relationships obtained from experiments of the type shown in Fig. 2. Each plot represents the average of 7–10 experiments. Error bars correspond to ±1 SD. (*A*), The results for a set of control cells and an averaged curve for 4 such sets of data is shown in the other panels: Ghosts were treated as described (*B*), with chymotrypsin to cleave the ankyrin bridges between spectrin and the transmembrane protein, band 3; (*C*), with PCMS to eliminate the actin network junctions; and (*D*), with NEM to dissociate spectrin tetramers. The curves correspond to the theory of Parker and Winlove (1999) using a value for C of 1000 and 9×10^{-27} Nm^{-1} for BH^2 for the unmodified cells, and 1.7×10^{-28}, 3.14×10^{-28}, and 1.3×10^{-28} for cells treated with chymotrypsin (*B*), PCMS (*C*), and NEM (*D*) (see Fig. 1 *A*.

Plastic yield of modified membranes

The formation of elliptocytes in the circulation when, in consequence of a genetic defect, a significant proportion of the spectrin is in the form of the dimer, implies that shape recovery after exposure to high shear (as when the cell passes through a capillary) may be incomplete. The resulting tendency for the cell to align itself with the long axis in the direction of fluid flow would ensure that this remains the preferred direction of stretching. To determine whether a yield phenomenon of this nature can be induced in cells in which a high proportion of spectrin dimers has been artificially generated, we applied prolonged stretches to the NEM-treated ghosts. The appearance of such a stretched cell is shown in Fig. 4. Whereas untreated control ghosts maintained tension for at least several minutes under a constant applied force of 20 pN, the treated cells exhibited a yield effect; this could be repeated many times on further stretching (Fig. 5). Averaged data for NEM-treated ghosts, compared to controls are shown in Fig. 6 *C*.

Both the chymotrypsin- and PCMS-treated ghosts behaved similarly to those treated with NEM and exhibited plastic yield under constant applied tension (Fig. 6, *A* and *B*).

Protein-free phospholipid vesicles

The extensibility of giant unilamellar lipid vesicles (Needham and Nunn, 1990; Käs and Sackmann, 1991) was tested. The vesicles were prepared from lipid of the same composition as the red cell membrane and are heterogeneous in size and shape, though in some cases approximately biconcave. Vesicles of the approximate size of a red cell were selected, and we found that beads coated with annexin V, which has a high calcium-dependent affinity for phosphatidylserine (Kuypers et al., 1996), would attach satisfactorily to the membrane surface. Sealed spherical vesicles, present in the preparations, could not (by reason of their already minimum surface:volume ratio in the osmotically

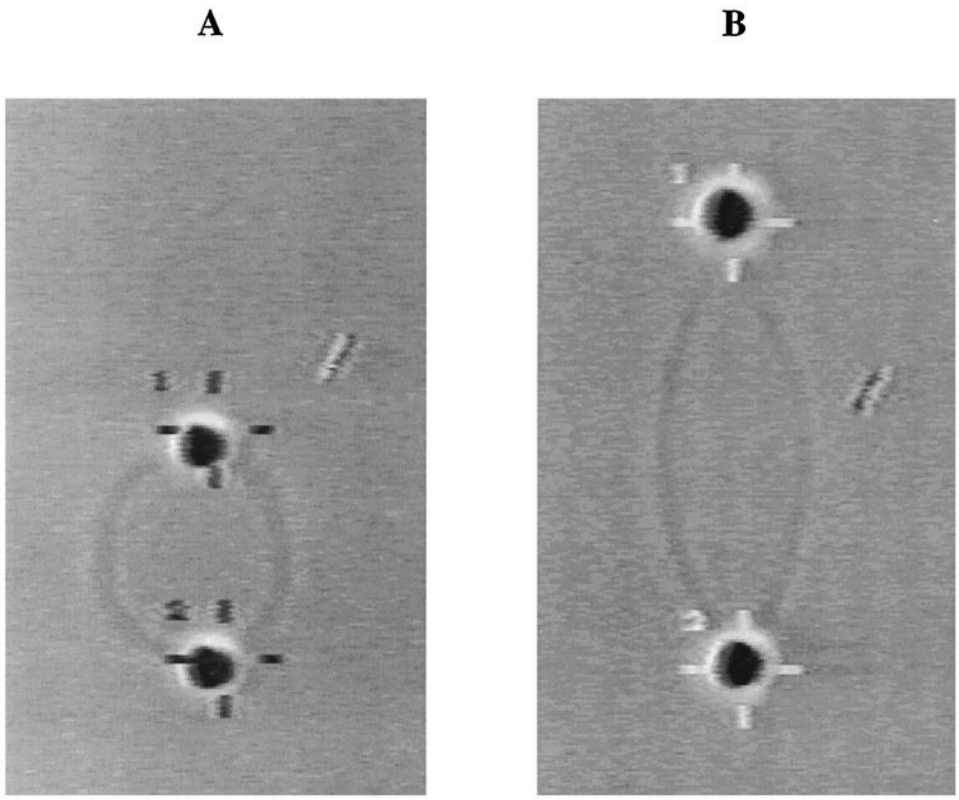

FIGURE 4 Appearance of NEM-modified red cell ghost in the optical trap, (*A*) before and (*B*) after yielding in response to a prolonged stretch.

inflated condition) be deformed at the forces generated by the optical trap. After treatment with saponin, however, the membranes of these vesicles became highly deformable. Because of their extreme softness and also the variation in size, no quantitative measurements of extension as a function of force were attempted, but comparison with ghosts and intact biconcave red cells left no room for doubt that the vesicle bilayer was very compliant and quite unlike the natural membrane. This observation agrees with the conclusions of micropipette analysis (Needham and Nunn, 1990). At the opposite extreme lie saponin ghosts, which have had their cytoskeletal protein networks cross-linked by treatment with glutaraldehyde: such cross-linked ghosts are completely inextensible by the optical tweezers.

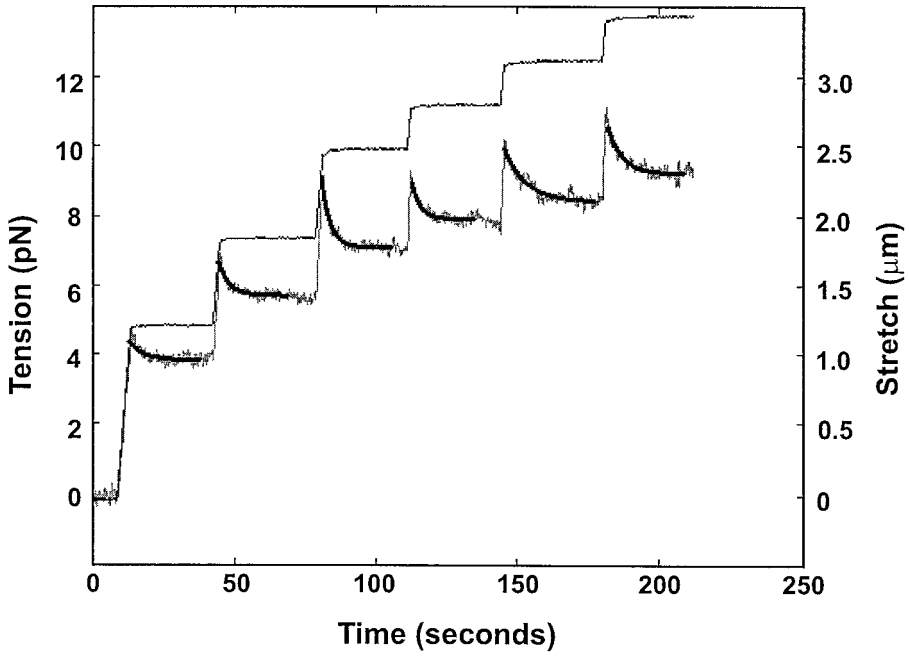

FIGURE 5 Response to prolonged stretches. A series of stretches were imposed on the ghost, which was allowed to relax back to a condition of near-equilibrium tension after each stretch. An exponential was fitted to the relaxation phases, the average rate being 0.1 s^{-1}. The quality of fit to such a single exponential and the rate were both somewhat variable between red cells modified in the three ways depicted in Fig. 1 *A*. Typical results are shown for a chymotrypsin-treated ghost. Untreated cells showed negligible relaxation.

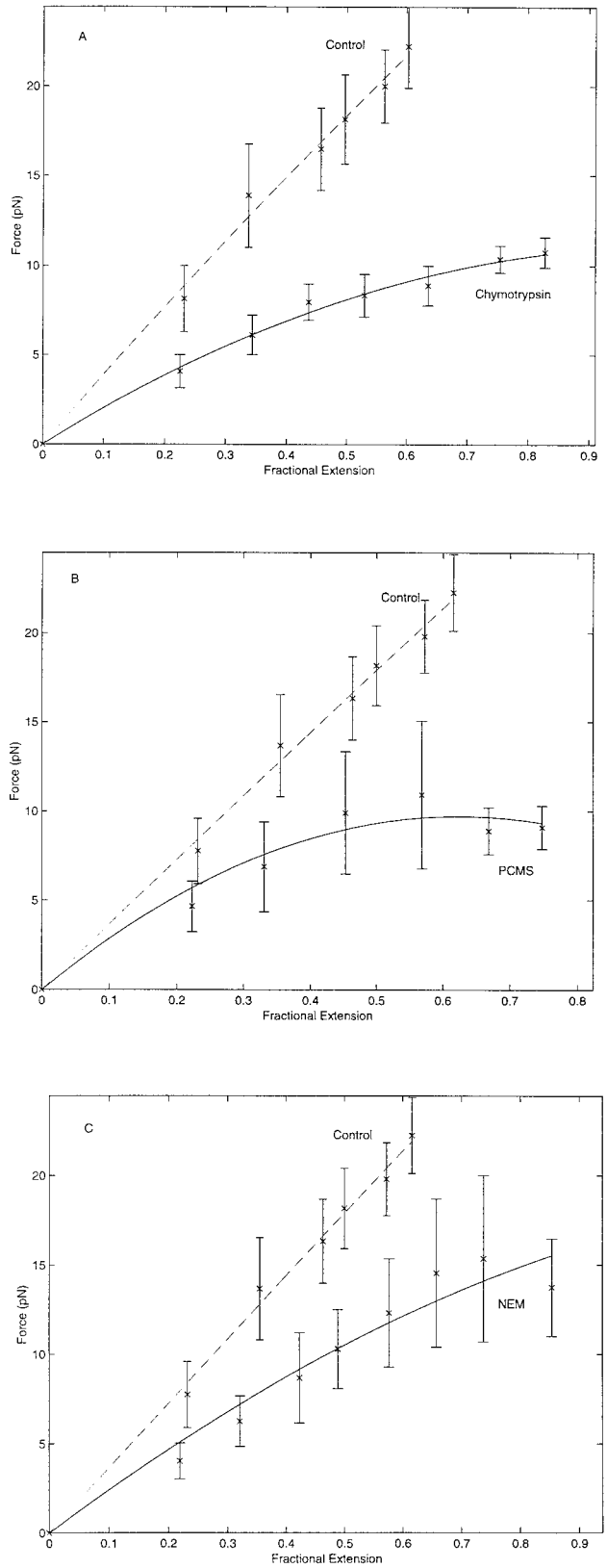

FIGURE 6 Force as a function of extension after relaxation of the cell to a near-equilibrium state, as shown in Fig 5. Profiles for ghosts, treated as indicated, with (*A*) chymotrypsin, (*B*) PCMS, and (*C*) NEM are compared in each case with averaged data for untreated control ghosts. The dashed line represents data for untreated control cells.

DISCUSSION

To allow the quantitative interpretation of force-extension relationships, using the theory for deformation of axisymmetric shells developed by Parker and Winlove (1999), we have studied saponin-permeabilized spherical ghosts. It seems likely that the elastic properties of these membranes differ little from those of intact red cells: we have observed, in the first place, that they exhibit force-extension profiles in much the same force regime (see also Hénon et al., 1999, discussed below). Moreover, saponin is a steroidal compound, analogous to cholesterol and known to act by binding to cholesterol in the membrane and not to lyse membranes that contain no cholesterol. Any saponin remaining in the membrane after lysis and extensive washing is unlikely to alter the membrane properties, since it has been shown that gross changes in the cholesterol content of the red cell membrane have no discernible effect on either the pressure-extension relation or the viscoelasticity, measured in the micropipette (Chabanel et al., 1983).

The theory developed in the accompanying paper (Parker and Winlove, 1999) predicts both the shape of the shell and the force as a function of polar extension for given values of the bending modulus B and the shear modulus H. Experimental constraints, however, allowed us to extract only the product BH^2 and not the individual moduli. The maximum force available in the optical tweezers was insufficient to engender a large enough distortion to permit modeling of the shape of the cell envelope. Moreover, the most sensitive region for the analysis of cell shape is that around the poles, where precise measurements are vitiated by the bead images. A more fundamental limit to interpretation of data at large strains may be that H does not remain invariant with ϵ. Our data, at all events, are confined, for the unmodified cells, to the small extension regime.

To proceed further, we need then to assume a literature value for either B or H, and we have chosen to put $B = 2 \times 10^{-19}$ Nm because this lies in the range given both by a micropipette method (Evans, 1983), which involves a much greater bending distortion than in our experiments, and by an analysis of the flicker phenomenon (Strey et al., 1995), in which the bending distortions are small. This value of B results in an estimate of 2×10^{-4} Nm^{-1} for H, which is an order of magnitude greater than the value given by micropipette methods (Evans and Hochmuth, 1978; Hochmuth and Hampel, 1979). A possible explanation emerges from the observation by Discher and Mohandas (1996) that large extensions pulled in the micropipette are accompanied by an effective phase separation, caused by the failure of the integral membrane proteins to follow the membrane flow and, instead, to accumulate at the entrance to the capillary.

Stokke et al. (1986a,b) have represented the red cell membrane as a composite of the lipid bilayer and an attached ionic protein gel, in which the spectrin tetramers function as entropy springs. Their model has the consequence that the pressure-extension relation in the micropipette should be determined by the (unknown) ratio of the

elastic shear modulus and the modulus of area compression of the cytoskeletal layer. In these circumstances, the shear modulus cannot be explicitly determined from the pressure-extension plot (Stokke et al., 1986b). The differences between our results and those of micropipette aspiration may then throw light on the origins of the elastic properties.

The modifications of the membrane-associated proteins cause a reduction in BH^2 by a factor of about 100. The literature values of B for cells (Evans, 1983; Strey et al., 1995) are close enough to those for protein-free lipid vesicles of similar size (Schneider et al., 1984; Faucon et al., 1989; Evans and Ravicz, 1990) to justify the assumption that B is unchanged by the modifications. Thus, the low value of BH^2 for the modified ghosts appears to be a consequence of a reduction of the shear modulus by almost an order of magnitude. The modifications do not change the elastic character of the cell, as observed in the optical tweezers, to anything near that of protein-free lipid membranes; we conclude that neither extensive interruptions of the membrane skeletal network nor scission of a primary attachment to the bilayer prevents the associated proteins from substantially increasing the shear elasticity of the membrane. The membrane skeletal constituent, band 4.1, remains after all the modifications and presumably retains its attachments to the membrane by way of the transmembrane protein, glycophorin C, and the peripheral protein p55 (see e.g., Hemming et al., 1995), but the binary association of spectrin with 4.1 is weak (Tyler et al., 1980) and, in any case, in the absence of actin protofilaments, there is nothing to retain the 4.1 molecules in the form of junctional clusters. It thus seems likely that the association per se of spectrin with the membrane is enough to render the material elastic. Our observations on cells in which the ankyrin has been severed imply that other interactions of spectrin, including that with the inner-leaflet anionic phospholipid, phosphatidylserine (see e.g., Maksymiw et al., 1987), may suffice to ensure a strong enough interaction to generate elasticity. It has to be recognized, however, that factors other than those we have considered here may influence the mechanical characteristics of the membrane. It has been suggested, for instance, that the interaction of the transmembrane protein, band 3, with lipid may be one such (Peters et al., 1996).

An obvious qualitative explanation for the elasticity of the red cell membrane is that the primary skeletal network constituent, the spectrin tetramer, functions, like the polymer of rubber, as an entropy spring: its configurational entropy is diminished by the restriction of its ends to a separation of about half the root-mean-square end-to-end distance of the molecule in free solution. The entropy-spring model for the network has been developed quantitatively (Kozlov and Markin, 1987; Boal, 1994), but does not provide the only possible basis of membrane elasticity. From a study of the changes in dimensions of isolated membrane skeletons as a function of temperature and medium composition, Vertessy and Steck (1989) suggested that the elasticity was determined by protein–protein interactions. McGough and Josephs (1990) inferred from the evidence of electron microscopy that the spectrin tetramers in the cytoskeletal lattice have the form of bihelical springs, which expand or contract without bending, in response to shear. Hansen et al. (1996) have developed a model that predicts the values of the shear modulus and the modulus of area expansion of a membrane with the network geometry of the red cell cytoskeleton, on the assumption that the spectrin tetramers behave in the manner suggested by McGough and Josephs and function as Hookean springs. Hansen et al. (1997a) further concluded that the deduced spring constant could not readily be accounted for by an entropy spring model because of insufficient flexibility of the spectrin molecule. The elastic properties of the model membrane were found (Hansen et al., 1997b) to be dependent on the network functionality (the average number of spectrin tetramers radiating from each network junction), and the analysis allows explicit predictions of how these properties would be expected to change when the functionality is altered, as in genetically abnormal cells.

At least for the case of the modified cells, in which the continuity or functionality of the network or its attachment to the bilayer is grossly disrupted, the elasticity of the membrane varies with applied force, and probably has more than one component. This behavior must also set limits on applicability of the analysis of Hansen et al. (1997b). They showed that their theory can explain the somewhat reduced shear modulus observed (Waugh and Agre, 1988) in hereditary spherocytes, characterized by a deficit of spectrin, if these cells embody a reduction in network functionality; however, this does not accord with the results of electron microscopy, which reveals a network of normal geometry in human (Liu et al., 1990) and mouse (Yi et al., 1997) spherocytes. The replacement of spectrin tetramers by dimers in cases of hereditary elliptocytosis, caused by mutations in the dimer–dimer association sites, was treated similarly by Hansen et al., and, as before, a decreased shear modulus is predicted; yet here again experimental micropipette measurements record an increase (Chabanel et al., 1989). This is also the case for cells treated, as in the present work, with NEM to dissociate 70% of the spectrin tetramers (Chabanel et al., 1989; Rangachari et al., 1989), where the theory predicts a diminution in shear modulus by two orders of magnitude. Hansen et al. (1997b) conclude that their elastic network model is inadequate to explain the nature of the elasticity of these cells. It may be noted that, for the case of an isotropic elastic network governed by entropy springs, the force required for a given extension also increases with increasing network functionality (Treloar, 1975). In our measurements, the stiffness of the membrane does indeed decrease with the loss of connecting lattice elements to about the extent demanded by the theory of Hansen et al. (1997b).

The plastic deformation of the modified membranes under tension affords an explanation for the elliptocytosis that invariably accompanies genetic anomalies associated with interruptions in the membrane skeletal network (Lux and Palek, 1995). In these cases, the asymmetric cell contour

must develop during physiological flow, which implies irreversibility of deformation. This may originate in failure of the shape to recover rapidly enough after a transient distortion, so that the asymmetry determines the direction of application of the next induced stress. It should be emphasized that our results do not necessarily bear on the stability of the membrane toward shearing forces, which is a property separable from its elastic characteristics (Chasis and Mohandas, 1986).

There have been three previous applications of optical tweezers to studies on red cells: Svoboda et al. (1992) used an optical trap to immobilize a cell while irrigating it with a nonionic detergent to liberate the membrane skeleton, and examined the contraction of the network with increasing ionic strength. Bronkhorst et al. (1995) used a multiple trap to measure rates of shape recovery of cells subjected to bending deformations. Most recently, and since the present work was submitted for publication, a further study has appeared, showing force-extension profiles for intact red cells (Hénon et al., 1999). Discocytic and osmotically swollen, nearly spherical cells were examined, and the data show that the extension for a given force of these cells is about twice what we observe on the permeabilized spherical ghosts. This is in good agreement with our own observations on unlysed dicocytes. Such a difference between the stretch response of flaccid intact cells and of the spherical membranes that we have studied is in accord with qualitative expectation. Hénon et al. (1999) derive from their results a shear modulus of 2×10^{-6} Nm^{-1}, which is two orders of magnitude lower than the value we have inferred from our data. Their analysis assumes that the biconcave cell can be treated as a planar disc and that their nearly spherical form as a sphere. Their treatment further implicitly assumes that the contribution of the bending stiffness of the membrane is negligible, whereas the exact solution of Parker and Winlove (1999) for an axisymmetric form implies that the force for a given extension is proportional to $B^{1/3}$. It is difficult to assess the validity of the assumptions made by Hénon et al. (1999), but we surmise that these, rather than any differences between the membranes of the intact and lysed cells, are responsible for the discrepancy between the conclusions. Our results suggest that the optical tweezers technique should have considerable advantages for the quantitative study of elasticity of membranes and cells.

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