TROPOMYOSIN MODULATES ERYTHROCYTE MEMBRANE STABILITY

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FOOTNOTES

The abbreviations used are: 4.1R, human erythrocyte protein 4.1; TM, tropomyosin; MgCl2, magnesium chloride; DI, deformability index; HRP, horseradish peroxidase; GST, glutathion-S-transferase.
ABSTRACT
The ternary complex of spectrin, actin and 4.1R defines the nodes of the erythrocyte membrane skeletal network, and is inseparable from membrane stability under mechanical stress. These junctions also contain tropomyosin (TM) and the other actin-binding proteins, adducin, protein 4.9, tropomodulin and a small proportion of capZ, the functions of which are poorly defined. Here we have examined the consequences of selective elimination of TM from the membrane. We have shown that the mechanical stability of the membranes of resealed ghosts devoid of TM is grossly, but reversibly impaired. That the decreased membrane stability of TM-depleted membranes is the result of destabilization of the ternary complex of the network junctions is demonstrated by the strongly facilitated entry into the junctions in situ of a β-spectrin peptide, containing the actin- and 4.1R-binding sites, after extraction of the TM. The stabilizing effect of TM is highly specific, in that it is only the endogenous isotype, and not the slightly longer muscle TM that can bind to the depleted membranes and restore their mechanical stability. These findings have enabled us identify a function for TM in elevating the mechanical stability of erythrocyte membranes by stabilizing the spectin-actin-4.1R junctional complex.

INTRODUCTION
The membrane of the erythrocyte represents an extreme case of shear resistance, imposed on the lipid bilayer by its associated skeletal protein network (1). In essence this network comprises a lattice of spectrin tetramers, formed by self-association of αβ spectrin heterodimers, attached at their ends to junctions consisting of short F-actin filaments (protofilaments), together with protein 4.1R, tropomyosin (TM) and several other actin-binding proteins, including adducin, protein 4.9 (dematin), a small proportion of capZ, and tropomodulin (2, 3). The actin filaments are, despite their short length (estimated as 12-15 subunits), resistant to dissociation by such agents as DNase and
cytochalasins (4, 5), and are rendered stable in this form by their interaction with several actin-binding proteins.

The elastic response of the cell to the large distortions that it experiences in the circulation derives from the network of elongated spectrin (α2β2) tetramers (1, 6) while the marked membrane stability results from the avidity of the spectrin dimer-dimer interaction and of the junctional complex of spectrin-actin-4.1R. Mutations in either α- or β-spectrin leading to defective tetramer formation and mutations in β-spectrin or 4.1R leading to a weakened junctional complex result in decreased membrane mechanical stability and cell fragmentation in vivo (7). While many insights have been garnered into our understanding of the role of spectrin and 4.1R in regulating membrane mechanical function, little is known about the contribution of other actin-binding proteins also present in the junctional complex to membrane function.

In the present study, we have sought to define more explicitly the contribution of TM to junctional stability. We have shown that selective depletion of TM from the membrane results in decreased membrane mechanical stability and that this effect is reversed when TM is restored. We have further shown that the decreased stability of TM-depleted membranes is the result of weakening of the ternary complex. These results identify a function for TM in elevating erythrocyte membrane stability by stabilizing the spectrin-actin-4.1R junctional complex.

EXPERIMENTAL PROCEDURES

Materials

Human venous blood was drawn from healthy volunteers with informed consent. Glutathione-Sepharose 4B was purchased from Amersham Pharmacia Biotech Inc (Piscataway, NJ). Dextran T40 from Amersham Pharmacia Biotech AB (Uppsala, Sweden), electrophoresis reagents from
Bio-Rad (Hercules, CA), GelCode Blue Reagent and Renaissance chemiluminescence detection kit from Pierce (Rockford, IL). Millipore Centriprep YM-30 was purchased from Fisher Scientific (Pittsburgh, PA). Anti-TM and anti-tropomodulin antibodies were kindly provided by Dr. V. M. Fowler (Scripps Institute, La Jolla), anti-adducin antibody by Dr. Y. Takakuwa (Tokyo Women’s Medical University, Tokyo) and anti-4.9 antibody by Dr. A. H. Chishti (University of Illinois, Chicago). Anti-4.1R antibody was generated and characterized in our laboratory. Anti-capZ antibody was from BD Biosciences Pharmingen (San Jose, CA). HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were from Jackson ImmunoResearch Laboratory (West Grove, PA). The approval to obtain fresh blood from healthy volunteers was obtained from the New York Blood Center institutional review board for these studies. Informed consent was approved according to the Declaration of Helsinki.

Methods

Preparation of Proteins. The recombinant fragment of the N-terminal region of human β-spectrin, comprising residues 1-301, was prepared by cloning and expression in Escherichia coli, as described by An et al. (8). Erythroid TM was purified from erythrocytes according to Fowler and Bennett (9) and muscle TM was purified according to Smillie (10). TM-containing erythrocyte membrane extract was prepared as follows: erythrocytes were washed three times in isotonic buffer followed by lysis and three washes in 15 volumes of magnesium-containing hypotonic buffer (5mM sodium phosphate, 1mM EGTA, 2 mM DTT, 2mM MgCl₂, 20μg/mL PMSF, pH 7.5). Ghosts were then washed once in a buffer devoid of magnesium ions (5mM sodium phosphate, 2 mM EDTA, 2 mM DTT, 20 μg/mL PMSF, pH 7.5) in order to extract TM. The resulting membranes were incubated for 90 min in 9 volumes of ice-cold distilled water containing 2 mM DTT and centrifuged for 30 minutes at 45,000 x g. The supernatant was collected and concentrated in a Centriprep YM-30 centrifugal filter. The presence of erythroid TM in the extract was
confirmed by Western blotting. The proteins were dialyzed against hypotonic buffer (5 mM Tris, 5 mM potassium chloride, pH 7.4) for incorporation into membranes. Protein concentrations were determined spectrophotometrically (11). Materials were screened for purity by gel electrophoresis.

**Preparation of Ghosts in the Absence or Presence of MgCl₂.** It is well established that magnesium depletion leads to selective loss of TM from erythrocyte membrane (9). This important finding provides us a means to study the potential role of TM. Thus, the erythrocytes were isolated from freshly drawn blood by centrifugation, and washed with Tris-buffered isotonic saline (0.15 M potassium chloride, 10 mM Tris, pH 7.4). The cells were then lysed and washed three times with 35 volumes of ice-cold hypotonic buffer (5 mM Tris, 5 mM potassium chloride, pH 7.4) without or with various concentrations of MgCl₂. To reseal the leaky ghosts, 0.1 volume of 1.5 M potassium chloride, 50 mM Tris, pH 7.4 was added to restore isotonicity and the ghosts were incubated for 40 min at 37 °C.

**Introduction of Protein into Erythrocyte Ghosts.** To examine the reversibility of the effect of magnesium depletion, purified erythroid TM, a TM-containing erythrocyte membrane extract or muscle TM was incubated with erythrocyte ghosts prepared without MgCl₂ for 10 min in the cold. The ghosts were resealed as described above, except that the resealing reaction contains 2 mM MgCl₂. The same procedure was used to introduce the β-spectrin N-terminal polypeptide 1-301 into ghosts prepared either in the absence or presence of MgCl₂. It needs to be pointed out that because MgCl₂ affects the size of pore and the dynamics of pore closure of the leaky ghosts (12), higher concentrations of peptides 1-301 were required to reach the same intracellular concentration. We determined the intracellular concentration of the peptide by SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) analysis of washed, resealed ghosts.
Measurement of Membrane Stability. To evaluate the effect of peptide incorporation on the resistance of the cells to mechanical shear, the resealed ghosts were suspended in 40% dextran, and examined in the ektacytometer, as described previously. The rate of decrease of deformability index (DI) at a constant applied shear stress of 750 dynes cm$^{-2}$ is a measure of membrane mechanical stability (13).

Membrane Skeleton Preparation from the Resealed Ghosts. The resealed ghosts were washed three times with isotonic buffer to eliminate residual free polypeptide from the external medium. The ghosts were extracted with 10 mM Tris, pH 7.0, 1% Triton X-100, 0.3M NaCl. The resulting membrane skeletons were washed and analyzed by 10% SDS-PAGE. The gel was stained with GelCode Blue and when required the relative concentrations of the protein components were assessed by densitometry.

Western Blots. 5 µg of packed erythrocyte ghosts were separated in 10% polyacrylamide gels and transferred onto nitrocellulose membrane. After blocking for 1 hour in blocking buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Tween-20, 5% non-fat dried milk powder), the blot was probed for 1 hour with the desired primary antibody (anti-4.1R, anti-adducin, anti-4.9, anti-tropomodulin, anti-capZ or anti-TM). After several washes, the blot was incubated with anti-mouse (or anti-rabbit) IgG coupled to HRP and developed with the Renaissance chemiluminescence detection kit. All steps were performed at room temperature.

RESULTS

Role of TM in Maintaining Membrane Mechanical Stability. The association of TM with F-actin requires divalent cations, which in the normal state of erythrocyte are solely magnesium, and indeed it is established that TM is lost from the membrane skeleton on washing with magnesium-
free buffer (9). This treatment does not cause dissociation, disproportion of the actin protofilaments, or any other known structural perturbation of the junctional complex (5, 14), although it does allow otherwise masked actin filament ends to act as nuclei for elongation by extraneous actin monomers (14). To determine whether the elimination of TM results in altered membrane mechanical function and any quantitative reduction in stability of the spectrin-actin complex we washed and incubated red cell ghosts in isotonic media containing no or varying concentrations of magnesium ions. Membrane mechanical stability was assessed by the resistance to shear in the ektacytometer (13). The signal (deformability index, DI) is produced by laser light scattered by the shear-deformed cells, which falls off when the membranes disintegrate into vesicles. Thus the rate of decay of the DI during shear is a direct measure of the capacity of the membranes to resist shear stresses. Fig. 1A reveals that membranes prepared in the absence of magnesium ions fragment rapidly when subjected to shear. Their stability rises with increasing magnesium concentrations in the initial preparation, approaching a plateau at a concentration similar to that in the native erythrocyte. Fig. 1B shows that while TM is lost from membranes prepared without magnesium, the amount of TM retained by the membrane skeletons increases with rising magnesium concentration, little or none being lost when this reaches 2 mM. Fig. 1C demonstrates that TM is the only recognized actin-binding protein liberated from the membrane skeletons when ghosts were prepared in the absence of magnesium. Immunoblots showed that, by contrast, 4.1R, tropomodulin and adducin, all of which have some form of filament-capping activity, remained with the membrane skeleton. So, also did the small fraction of erythrocyte capZ (the bulk of which is cytoplasmic (15)), and the actin-bundling species, protein 4.9.

Reversal of Effects of Magnesium Depletion and Specificity of erythroid TM. To determine whether the effect of magnesium depletion on membrane stability is due to magnesium itself or to the loss of TM, we first tested whether the effect could be reversed by the addition of magnesium
chloride to TM-depleted membranes and found that it had no effect on the DI or its rate of decay with time (data not shown). When, on the other hand, purified erythroid TM was added, together with 2 mM magnesium ions, to ghosts from which magnesium and therefore erythroid TM had been eliminated, the shear resistance was partially restored (profile 3 of Fig. 2A). Since the erythroid TM preparation involves a harsh heating step, and high concentrations of the renatured proteins are not readily obtained because of the limited solubility, we also examined the recovery of shear resistance on addition of the unfractionated extract containing erythroid TM as a major constituent, presumably largely in the native state. As shown in Fig. 2A (profile 4), the shear resistance was almost completely recovered, as indicated by the initial slope of the fragmentation curve. The restoration of membrane stability parallels the rebinding of erythroid TM to the membranes (Fig 2B). Furthermore, the restoration by erythroid TM is highly specific because addition of purified muscle TM to the depleted cells was without effect on their shear resistance (Fig 2C), and immunoblots revealed that it did not bind perceptibly to the membranes (Fig 2D).

This was not a consequence of an intrinsic inability of muscle TM to bind to the β-actin isotype of erythrocyte, for we found that F-actin of platelets, with the identical sequence, bound to muscle TM as effectively as erythroid TM in a pelleting assay (data not shown).

**Destabilization of Spectrin-Actin-4.1R Ternary Complex on Elimination of TM.** To explore the mechanism by which TM affects membrane mechanical stability, we examined whether the ternary complex of spectrin, F-actin and 4.1R, that characterizes the junctions of the membrane skeleton, is rendered more labile by the removal of TM. We introduced into ghosts a GST-linked N-terminal fragment (residues 1-301) of the spectrin β-chain. This peptide encompasses the binding region for actin and 4.1R of the ternary complex and has been shown to disrupt spectrin-actin-4.1R ternary complex formation in vitro (8). On incubation, the peptide was incorporated into the membrane skeleton (Fig 3A). Concomitantly the shear-resistance of the membranes, as assayed in the
ektacytometer, was grossly degraded (Fig 3B). GST was without effect. Thus it appears that the peptide competes with the endogenous spectrin in generating the ternary complex, and that the ensuing dissociation of spectrin from the junctions destabilizes the membrane. When ghosts were prepared in the presence of magnesium, the incorporation of this peptide into membrane skeleton was reduced. The apparent association constant of the peptide with membranes devoid of TM was found to be $3.9(\pm 0.6) \times 10^4 M^{-1}$, and $2.0(\pm 0.2) \times 10^4 M^{-1}$ when TM remained bound. Consequently, the peptide at given concentration exhibited a smaller destabilizing effect on ghosts prepared in the presence of magnesium than on those depleted of erythroid TM by exposure to magnesium-free buffer (Fig 3D).

DISCUSSION

The present findings enabled us to identify a function for erythroid TM in enhancing the shear resistance of the membrane by stabilizing the spectrin-actin junctional complex. The TM is present in the cell at about the theoretical saturating concentration, namely some two molecules for each actin filament with an estimated length of 12-15 subunits (9). It is evidently not required to ensure the survival of the actin in its unusually short filamentous form. While proteolysis of other membrane skeletal proteins, in particular presumably spectrin and 4.1R, permits the disproportion of the short into long filaments of F-actin (16, 17), loss of TM on removal of magnesium does not lead to any sensible diminution in the number of filament ends or therefore of filaments (5, 14). An observed change, however, that results from the elimination of magnesium ions is the exposure of filament ends, which are normally capped (14), although whether this is a direct consequence of the loss of TM is uncertain. There is in any event an unaccountable abundance of capping proteins in the erythrocyte, none of them needed to prevent depolymerization. Positive-end (fast-growing end) capping proteins include adducin (18) and erythrocyte capZ (15), while the negative-ends may be capped by tropomodulin (19, 20) and by spectrin-4.1R (21). There is, besides, at least one other
actin-binding protein in the membrane, protein 4.9, or dematin, which has filament-bundling activity \textit{in vitro} (22). What purpose the multiplicity of capping systems serve in the red cell is unclear; except now for TM, to the extent at least that we have indicated.

The observation that muscle TM cannot substitute for its erythroid analogue is unexpected. A remarkable difference between the two proteins is that, while both interact with erythroid tropomodulin, and with similar affinities, they bind to different sites on the tropomodulin (23). This could be taken to imply that tropomodulin participates in the TM-dependent stabilization of the junctional complexes. On the other hand, our observation that, while muscle TM does not bind to the short $\beta$-actin filaments in the membrane, it does bind to long filaments of the same isotype offers an alternative explanation. Muscle TM has 284 amino acid residues, and two such molecules will cover 14 subunits in an F-actin filament. By contrast, erythroid (5a or 5b) tropomyosin contains 248 residues, and must therefore cover fewer (probably 12) subunits. The F-actin double-helix has 13 monomers per turn of 35.5 nm, whereas the length of the filaments in the red cell has been estimated from electron microscopy (24) as 33.37 nm. Thus the possibility exists that inability of muscle TM to bind to these protofilaments is due to a mismatch of lengths. If this is so, it would follow that the length of the protofilaments is tightly regulated, and the TM acts as a length regulator during assembly, as Fowler (25) has suggested.

The capacity of the N-terminal $\beta$-spectrin fragment to enter the junction complexes signifies that these are more labile than had been supposed, even in the presence of TM. The ensuing impairment of shear-resistance is comparable to that observed in hereditary elliptocytes resulting from 4.1R deficiency (26), which may also be presumed to have junctions of impaired stability. Whether one can infer that the network junctions are normally dynamic, in the sense of allowing transient spectrin dissociation when the membranes undergo distortion at physiological shearing stresses is
not yet clear. If this turns out to be the case, it will add to the known or surmised mechanisms by which the membrane can respond to large distortions, namely extension of the spectrin chains to their full contour lengths (27), unfolding of some of the triple-helical structural repeats that make up the bulk of the spectrin chains (28-31) and dissociation of the spectrin tetramers that link the junction complexes into dimers (32).
REFERENCES


15. Kuhlman PA, Fowler VM. Purification and characterization of an alpha 1 beta 2 isoform of CapZ from human erythrocytes: cytosolic location and inability to bind to Mg2+ ghosts suggest that erythrocyte actin filaments are capped by adducin. Biochemistry. 1997;36:13461-72.


FIGURE LEGENDS

**Fig. 1** Effect of Magnesium Depletion on Membrane Stability and on Membrane Protein Retention. **A:** Membrane stability, expressed as the rate of decline in ektacytometric deformability index (DI), increases (decay curve shifted towards right) with increasing concentrations of MgCl₂ maintained during preparation of the ghosts. **B:** Retention of TM by ghosts examined in A. **C:** Components of junction complexes of ghosts prepared in the absence or in the presence of 2 mM MgCl₂. The panels show immunoblots after SDS-PAGE of total protein from ghosts, prepared with and without MgCl₂ as indicated, probed with antibodies against 4.1R, adducin, protein 4.9, capZ, tropomodulin and TM.

**Fig. 2** Reversibility of the Effects of Magnesium Depletion. **A:** Membrane fragmentation profiles of ghosts prepared in the presence of 2 mM MgCl₂ (1); ghosts prepared in the absence of MgCl₂ (2); TM-depleted ghosts after addition of purified erythroid TM (3); TM-depleted ghosts after addition of a membrane extract containing TM (4). Note the partial restoration of membrane stability by purified erythrocyte TM and almost complete restoration when the extract was added. **B:** Rebinding of erythroid TM to membrane preparations shown in Panel A documented by immunoblotting with anti-TM antibody. **C:** Membrane stability, of ghosts prepared in the presence of MgCl₂ (1); in the absence of MgCl₂ (2); and after addition of purified muscle TM (3) showing that this fails to increase membrane stability. **D:** Failure of rebinding of muscle TM to membrane preparations shown in Panel C documented by immunoblotting with anti-TM antibody. Lane 4, purified muscle TM used for membrane reconstitution.
Fig. 3 Incorporation of Polypeptide 1-301 of β-Spectrin into Membrane Skeletal Junctions in situ and Effect of the Incorporation on Membrane Stability. A: GST-tagged polypeptide 1-301 was introduced into TM-depleted ghosts. Isolated membrane skeletons from the resealed ghosts were analyzed by electrophoresis in 10% SDS-PAGE. B: Effect of polypeptide 1-301 on membrane stability of TM-depleted ghosts. Membrane stability, expressed as the rate of decline in DI, diminishes (decay curve shifted towards left) with increasing concentration of peptide. C: Binding of the peptide to ghost membranes, prepared in the absence (filled circles) and presence (open circles) of magnesium ions. The curves are calculated best-fits for a single set of independent spectrin-4.1R binding sites, assuming a site concentration of 1.2 µM. The resulting values of $K_a$ are $3.9 (± 0.6) \times 10^4$ (broken line) and $2.0 (± 0.2) \times 10^4$ M$^{-1}$ (full line). D: Effect of incorporated peptide on shear-resistance of ghosts prepared in the absence (filled circles) or presence (open circles) of 2 mM MgCl$_2$, showing reduced shear resistance in the former. Membrane stability was assessed from DI decay curves. The relative stability is defined as the ratio of time under shear for a 50% drop in signal to that for TM-containing ghosts at zero peptide concentration.
Fig 1

A

B

C

MgCl₂ (mM)

Deformability Index

Time (second)

0 50 100 150 200 250

0.0 0.2 0.4 0.6 0.8

MgCl₂ (mM)

0 0.05 0.25 0.50 2.00

TM

4.1R

tropomodulin

adducin

CapZ

4.9

TM

- MgCl₂

+ MgCl₂

- MgCl₂

+ MgCl₂
Fig. 3

A

spectrin
4.1R
GST-1-201
actin

Peptide concentration inside the ghosts (μM)

B

Time (seconds)

control
5 μM
10 μM
20 μM
40 μM
50 μM

D

Relative Membrane Stability

Peptide concentration inside the ghosts (μM)

Peptide concentration incorporated (μM)

0.9
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

0
10
20
30
40
50
60

0
10
20
30
40
Tropomyosin modulates erythrocyte membrane stability

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