Tropomyosin modulates erythrocyte membrane stability

Xiuli An,1 Marcela Salomao,1 Xinhua Guo,1 Walter Gratzer,2 and Narla Mohandas1

1Red Cell Physiology Laboratory, New York Blood Center, New York, NY; 2Kings College London, Randall Center for Molecular Mechanisms of Cell Function, Guy’s Campus, London, United Kingdom

The ternary complex of spectrin, actin, and 4.1R (human erythrocyte protein 4.1) 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 spectrin-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 (human erythrocyte protein 4.1), 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.

Materials and methods

Materials

Human venous blood was drawn from healthy volunteers with informed consent. Glutathione-Sepharose 4B was purchased from Amersham Pharmacia Biotech (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); and Millipore Centriprep YM-30, from Fisher Scientific (Pittsburgh, PA). Anti-TM and antitropomodulin antibodies were kindly provided by Dr V. M. Fowler (Scripps Institute, La Jolla, CA); antiadducin antibody, by Dr Y. Takakuwa (Tokyo Women’s Medical University, Tokyo, Japan); and anti-4.9 antibody, by Dr A. H. Chishti (University of Illinois, Chicago, IL). Anti-4.1R antibody was generated and characterized in our laboratory. Anti-capZ antibody was from BD Biosciences Pharmingen (San Jose, CA). Horseradish peroxidase (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.

© 2007 by The American Society of Hematology
Methods

Preparation of proteins. The recombinant fragment of the N-terminal region of human β-spectrin, comprising residues 1 to 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 3 times in isotonic buffer followed by lysis and 3 washes in 15 volumes of magnesium-containing hypotonic buffer (5 mM sodium phosphate, 1 mM EGTA, 2 mM DTT, 2 mM MgCl2 [magnesium chloride], 20 μg/mL PMSF, pH 7.5). Ghosts were then washed once in a buffer devoid of magnesium ions (5 mM sodium phosphate, 2 mM EDTA, 2 mM DTT, 20 μg/mL PMSF, pH 7.5) and then washed twice in 5 volumes of magnesium-containing hypotonic buffer (5 mM Tris, 5 mM potassium chloride, pH 7.4) for incorporation into membranes. Protein concentrations were determined spectrophotometrically.

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 3 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 minutes 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 minutes in the cold. The ghosts were resealed as described above under “Preparation of ghosts in the absence or presence of MgCl2,” 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 pores 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 sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) 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 3 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. Packed erythrocyte ghosts (5 μg) 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% nonfat dried milk powder), the blot was probed for 1 hour with the desired primary antibody (anti-4.1R, antiadducin, anti-4.9, antitropomodulin, 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 the erythrocyte are solely magnesium cations, 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,5,24 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, or 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. Figure 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. Figure 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. Figure 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. Also remaining were the small fraction of erythrocyte capZ (the bulk of which is cytoplasmic) 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 Figure 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 unfraccionated extract containing erythroid TM as a major constituent, presumably largely in the native state. As shown in Figure 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 (Figure 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 (Figure 2C), and immunoblots revealed that it did not bind perceptibly to the membranes (Figure 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, which characterizes the junctions of the membrane skeleton, is rendered more labile by the removal of TM. We introduced into ghosts a glutathione-S-transferase (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. On incubation, the peptide was incorporated into the membrane skeleton (Figure 3A). Concomitantly, the shear resistance of the membranes, as assayed in the ektacytometer, was grossly degraded (Figure 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 $3.9 \times 10^4$ M$^{-1}$ ($=0.6 \times 10^4$ M$^{-1}$) and $2.0 \times 10^4$ M$^{-1}$ ($=0.2 \times 10^4$ 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 (Figure 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 2 molecules for each actin filament with an estimated length of 12 to 15 subunits. An observed change, however, that results from the elimination of magnesium ions is the exposure of filament ends or therefore of filaments. An observed change, however, that results from the elimination of magnesium ions is the exposure of filament ends, namely some 2 molecules for each actin filament with an estimated length of 12 to 15 subunits. 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, loss of TM on removal of magnesium does not lead to any sensible diminution in the number of filament ends or therefore of filaments. An observed change, however, that results from the elimination of magnesium ions is the exposure of filament ends, which are normally capped, 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 and erythrocyte capZ, while the negative ends may be capped by tropomodulin and by spectrin-4.1R. There is, however, at least one other actin-binding protein in the membrane, protein 4.9, or dematin, which has filament-bundling activity in vitro. What purpose the multiplicity of capping systems serves 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 analog is unexpected. A remarkable difference between the 2 proteins is that, while both interact with erythroid tropomodulin, and with similar affinities, they bind to different sites on the tropomodulin. 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 β-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 2 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 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 has suggested.

The capacity of the N-terminal β-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 with that observed in hereditary elliptocytes resulting from 4.1R deficiency, 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, unfolding of some of the triple-helical structural repeats that make up the bulk of the spectrin chains, and dissociation of the spectrin tetramers that link the junction complexes into dimers.

**Acknowledgments**

This work was supported in part by NIH grants DK 26263, DK 32094, and HL31579.

**Authorship**

Contribution: X.A. designed experiments, analyzed the data, and wrote the paper; M.S. and X.G. performed research and analyzed the data; W.G. and N.M. designed experiments, analyzed the data, and edited the paper. X.A. and M.S. contributed equally to this work.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Xiuli An, Red Cell Physiology Laboratory, 310 E 67th St, New York, NY 10021; e-mail: xan@nybloodcenter.org.

**References**


11. Perkins SJ. Protein volumes and hydration ef-ficiences, electron microscopy 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 has suggested.


15. Kuhlman PA, Fowler VM. Purification and charac-terization 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 addu-cin. Biochemistry. 1997;36:13461-13472.


Tropomyosin modulates erythrocyte membrane stability

Xiuli An, Marcela Salomao, Xinhua Guo, Walter Gratzer and Narla Mohandas