Immunolocalization of tropomodulin, tropomyosin and actin in spread human erythrocyte skeletons

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SUMMARY

The human erythrocyte membrane skeleton consists of a network of short actin filaments cross-linked into a hexagonal network by long, flexible spectrin molecules. The lengths of the short actin filaments (33±5 nm) at the central junctions are proposed to be stabilized and limited by association with tropomyosin and the tropomyosin-binding protein, tropomodulin. Here, we use immunogold labelling followed by negative staining to specifically localize tropomodulin, tropomyosin and actin to the sites of the central junctions in spread membrane skeletons. In addition to negative staining, immunogold labelling for tropomodulin at the sites of the central junctions was also visualized by a quick-freeze, deep-etch, rotary-replication technique. These experiments confirm previous indirect evidence that the short filaments at the central junctions are indeed actin filaments and provide the first direct evidence that tropomodulin and tropomyosin are associated with the erythrocyte actin filaments in situ.

Key words: tropomodulin, tropomyosin, erythrocytes

INTRODUCTION

The biconcave shape, mechanical strength and deformability properties of the human erythrocyte are determined by a complex membrane-associated skeleton consisting of spectrin, actin and numerous spectrin- and actin-binding proteins (ankyrin, protein 4.1, adducin, dematin, tropomyosin, tropomodulin; for reviews, see Branton et al., 1981; Bennett, 1985, 1989; Cohen, 1983). Platinum replica methodology has revealed the membrane-associated skeleton to be a dense, intricate network of filaments in situ (Coleman et al., 1989; Ursitti et al., 1991), making it difficult to dissect the organization of the proteins by structural methods. As a result, models for the structural organization of the membrane skeleton were originally derived from extensive biochemical and morphological analysis of purified proteins in vitro (Branton et al., 1981; Cohen, 1983). Remarkably, a few years ago, the conclusions and models derived from these biochemical experiments were substantiated by negative staining electron microscopy of isolated, spread membrane skeletons (Byers and Branton, 1985; Liu et al., 1987; Shen et al., 1984, 1986). These images revealed a regular, predominantly hexagonal network of 200 nm long spectrin filaments arranged as spokes around central, thicker, 33±5 nm long actin filament hubs (referred to as central junctions); between 5 and 7 spectrin molecules were attached to each actin filament. The spectrin molecules were identified based on their similar morphological appearance to purified spectrin tetramers (Shotton et al., 1979) and the 33 nm long filaments at the junctions were identified indirectly as actin based on their ability to polymerize actin monomers from one end (Byers and Branton, 1985; Shen et al., 1984). The orientation of the spectrin molecules with respect to the actin filaments (i.e. tails of the spectrin dimers attached to the actin filaments) was demonstrated by direct immunogold labelling of specific regions along the spectrin molecules using domain-specific monoclonal antibodies (Ursitti and Wade, 1993). In addition, immunogold labelling of isolated membrane skeletons localized ankyrin to the globular structure observed near the center of the spectrin filaments (i.e. at the heads of each spectrin dimer; Derick et al., 1992; Liu et al., 1987).

In addition to spectrin, several other components of the membrane skeleton have been demonstrated to bind actin filaments in vitro and therefore were predicted to be associated with the short actin filaments in the membrane skeleton in situ. These include protein 4.1, which binds to the N terminus of the spectrin β-subunit (tail end of the spectrin dimer) and forms a ternary complex with spectrin and actin; adducin, which promotes spectrin binding to actin and bundles actin filaments; and dematin, which also bundles actin filaments (for a review, see Bennett, 1989). Again, immunogold labelling of isolated skeletons has localized protein 4.1, adducin and dematin to the central actin junction (Derick et al., 1992). Two actin-associated proteins in the membrane skeleton that remain to be localized directly are tropomyosin and tropomodulin.

Tropomyosin (TM) is a rod-like, α-helical dimer that binds along the length of actin filaments in muscle and nonmuscle cells. Two nonmuscle isoforms of TM with subunit molecular masses of 27 and 29 kDa are associated with erythrocyte
membranes prepared by lysis in the presence of millimolar concentrations of magnesium (Fowler and Bennett, 1984a). In vitro, TM binding to pure actin filaments also requires millimolar magnesium concentrations (Fowler and Bennett, 1984a), supporting the idea that TM is associated with the short actin filaments on the membrane. Furthermore, stoichiometric analysis indicates that there are sufficient levels of TM in Mg$^{2+}$-ghosts to coat each short actin filament with two 33-34 nm long TM molecules, one on each side of the 33±5 nm long actin filaments (Fowler and Bennett, 1984a; Shen et al., 1986).

TM has been proposed to stabilize the short actin filaments in the erythrocyte membrane and to regulate the number of spectrin molecules attached to each short filament (Fowler and Bennett, 1984a,b; Shen et al., 1986).

Tropomodulin (Tmod) is a 40.6 kDa membrane skeleton protein that binds to one end of TM and is present in approximately equimolar amounts with respect to TM in the membrane skeleton (Fowler, 1987; Fowler, 1990; Sung et al., 1992). Tmod is also present in skeletal muscle and is located at the pointed (slow growing) ends of the thin filaments in muscle sarcomeres (Fowler et al., 1993). Recently, we have shown that Tmod prevents elongation and depolymerization of actin filaments from their pointed ends in vitro, suggesting that Tmod functions to limit the lengths of the short erythrocyte actin filaments as well as the long muscle actin filaments by capping their pointed ends (A. Weber, C. Pennise, G. Babcock and V. Fowler, unpublished observations).

In this paper, we have used immunogold labelling of isolated, spread membrane skeletons to provide direct evidence that the short 33 nm filaments at the central junctions are indeed actin and that TM and Tmod are localized on these short actin filaments. Our results further support the hypothesis that TM and Tmod function together to stabilize and limit the lengths of the short actin filaments in the erythrocyte membrane skeleton.

**MATERIALS AND METHODS**

**Skeleton preparation**

Blood was drawn from normal individuals into sterile tubes containing lithium heparin. Blood was either used immediately or stored at 4°C and used within 24 hours of collection. Pink ghosts were prepared by lysing washed red blood cells with 5 mM sodium phosphate (NaPi) buffer (pH 7.4; Dodge et al., 1962) with additions of 1 mM EGTA and 2 mM MgCl₂. PMSF was added fresh to the first two washes at a concentration of 20 µg/ml (Fowler and Bennett, 1984a). Skeletons were isolated as previously described (Shen et al., 1984). Briefly, pink ghosts were incubated on ice for 1 hour in 4 vols of lysis buffer containing 2.5% Triton X-100. The mixture was layered on top of a discontinuous density gradient of 10/35/60% sucrose (w/v) with 0.1 mM NaPi (pH 8.0), 2 mM MgCl₂, and 0.5 mM dithiothreitol (DTT). The gradients were centrifuged in a Beckman SW-40 rotor at 70,000 rpm for 20 minutes at 4°C. Skeletons were collected from the 35/60% sucrose interface and dialyzed overnight against 2 mM NaPi, pH 7.4, 1 mM EGTA, 2 mM MgCl₂, and 0.5 mM DTT. Dialyzed skeletons were diluted with 9 vols of 0.1 mM NaPi and incubated for 1-2 hours on ice.

**EM grid preparation**

For negatively stained images, thin carbon films were used to obtain highest resolution. Carbon films were produced by evaporating a small amount of carbon from the electron beam evaporation gun in a Balzers 300 freeze-etch apparatus onto freshly cleaved squares of mica. The carbon film was floated off the mica squares onto a clean distilled water surface and picked up with 600-mesh nickel grids (Polysciences, Warrington, PA). For quick freeze, deep-etch rotary replication (QFDERR), grids were first coated with a thin film of Formvar followed by evaporation of carbon onto the Formvar surface. All grids were glow discharged for 1 minute in the chamber of the Balzers apparatus just before use.

**Sample preparation and immunogold labelling**

Skeletions were immobilized by dripping ~3 ml of diluted skeletons onto each glow discharged grid at 4°C. The grids were rinsed with 0.1 mM NaPi, pH 8.0. Unless indicated otherwise, all washes and incubations were carried out in a 100 µl drop of solution. Samples were fixed with a solution of 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mM NaPi, pH 8.0 (2% PF/0.1% GT) for 15 minutes at room temperature (RT). After fixation, the grids were rinsed in 0.1 mM NaPi (pH 8.0). Aldehyde groups were blocked by incubating the grids at RT in 50 mM NH₄Cl in phosphate buffered saline (PBS) for 15 minutes, transferring the grid to a fresh drop after 7.5 minutes. Nonspecific binding was blocked by incubating the carbon-coated grids in 0.1% immunogold silver staining quality gelatin (IGSS gelatin; Amersham Corporation, Arlington Heights, IL) in PBS for 30 minutes at RT. The grids were transferred to fresh solution after 15 minutes. Nonspecific binding to samples supported on Formvar/carbon grids was blocked with a solution of 1 mg/ml BSA, 3% normal goat serum, and 0.1% IGSS gelatin in PBS. Primary antibodies were diluted to 10-20 µg/ml in PBS plus the appropriate blocking reagents. The grids were incubated in 20 µl of the diluted primary antibodies for 2-4 hours at RT. After primary antibody incubation, the grids were washed three times for 10 minutes each in Tris buffered saline (TBS; 150 mM NaCl, 20 mM Tris, 10 mM Na₂SO₄, pH 7.4) at 4°C with 0.1% IGSS gelatin added for incubations on carbon-coated grids or 1% BSA added for incubations on Formvar/carbon grids. The secondary antibodies, either goat anti-rabbit IgG adsorbed to 5 nm gold particles (Ted Pella, Inc., Redding, CA) or goat anti-rabbit IgG adsorbed to 10 nm gold particles (Amersham Corporation; Arlington Heights, IL), were diluted 1:40 in the appropriate solution just before use. The grids were incubated in 20 µl of diluted gold-adsorbed antibody for 2 hours at RT. Both types of grids were washed three times for 10 minutes each in 0.1% gelatin in PBS. The gelatin was then removed from the grids by washing for 1 minute in 0.05% Triton X-100 in PBS, followed by four 1 minute washes in PBS. The grids were then fixed at RT for 15 minutes in 2% PF/0.1% GT. The fixative was rinsed from the grids with 0.1 mM NaPi. Immunolabelled skeletons on carbon-coated grids were negatively stained with aqueous 1% uranyl acetate and allowed to air dry in a covered Petri dish. The procedure for immunonegative staining has been described previously for viruses (Hayat, 1989) and human erythrocyte skeletons (Derick et al., 1992; Ursitti and Wade, 1993).

**Antibodies**

Antibodies to human erythrocyte tropomodulin were generated in rabbits and affinity-purified as described (Fowler, 1990). Antibodies to human erythrocyte tropomyosin were generated in rabbits by injection of tropomyosin coupled to keyhole limpet hemocyanin at a ratio of 1:1 tropomyosin:hemocyanin (mol/mol, using a molecular mass of 60,000 for tropomyosin and 100,000 for hemocyanin), as described by Kreis (1986). Tropomyosin was purified as described (Fowler and Bennett, 1984a), with two additional steps of chromatography on hydroxyapatite (Cote and Smillie, 1981) followed by sedimentation on a 5-20% sucrose gradient to remove minor amounts of high molecular mass contaminants. Tropomyosin antibodies were affinity-purified from immune sera over a column of erythrocyte tropomyosin coupled to cyanogen bromide-activated Sepharose (Cl 4B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) by standard procedures, eluting with 0.2 M glycine-HCl, pH 2.8. The anti-
Fig. 1. Immunogold labelling of actin filaments, tropomyosin and tropomodulin in isolated human erythrocyte skeletons visualized by negative staining. Primary antibodies to (A) β-actin, (B) tropomodulin, and (C) tropomyosin localize to the central junctions in spread and negatively stained skeletons. Skeletons are not labelled when incubated with preimmune rabbit IgG (D). Immunogold labelling with secondary antibodies adsorbed to 5 nm (B and C) and 10 nm (A and D) gold particles was performed after incubation with the primary antibodies listed above. Arrowheads point to immunogold-labelled central junctions. Note, multiple gold particles are frequently observed associated with a single central junction. This is likely to be due to the presence of more than one Tmod, TM or actin molecule at each junction, multiple primary antibody binding actin sites on an individual molecule, and/or to multiple secondary antibody binding sites on the primary antibodies.
bodies used in this paper were a generous gift from Dr J. C. Bulinski (Columbia University College of Physicians and Surgeons, New York, NY). An affinity-purified antibody prepared against the N terminus of β-actin (Otey et al., 1987) was used for immunoelectron microscopic labelling procedures, and antiserum prepared against the N terminus of skeletal muscle α-actin (Miller et al., 1987), which also recognizes β-actin, was used for immunoblotting procedures. Antibodies to β-actin were also kindly provided by Dr I. M. Herman (Tufts University, Boston, MA) and Dr D. W. Speicher (The Wistar Institute, Philadelphia, PA). All of these actin antibodies were found to yield essentially the same results.

**Quick-freeze, deep-etch, rotary replication (QFDERR)**

The QFDERR procedure has been described previously (Ursitti and Wade, 1993). Briefly, grids were rinsed in filtered HPLC-quality water and secured in the forceps of a plunge-freezing apparatus. The level of water on the sample was adjusted just before dropping the sample into a mixture of propane and 25% isopentane that was cooled with liquid N2. The frozen grids were clamped into a 6-place sample holder. Etching and replication were performed in a Balzers freeze-etch unit. Samples were etched at −95°C for 3 hours and then rotary-replicated with Pt at an angle of 20°; carbon was not applied. Film thickness was estimated by the frequency shift on the Balzers quartz crystal monitor (Tyler and Branton, 1980). All electron microscopy specimens were viewed in a Zeiss EM 10CA operating at 80 kV.

**Electrophoresis procedures**

Samples were electrophoresed on 7.5-15% linear gradient SDS-polyacrylamide gels with a 5% stacking gel as described (Laemmli, 1970), except that the pH of the gradient gel was 8.6 (Fowler, 1990). After electrophoresis, gels were stained with protein with Coomassie Brilliant Blue R250 or were electrophoretically transferred to nitrocellulose paper (0.2 µm; Schleicher & Schuell, Inc., Keene, NH) as previously described (Fowler, 1987). Transfers were labelled with affinity-purified antibodies to erythrocyte tropomodulin or tropomyosin (1 µg/ml) or anti-actin (1:1000) followed by 125I-Protein A (Fowler, 1990). Labelled polypeptides were visualized by exposure to X-ray film at −80°C (XAR-5; Eastman Kodak Co., Rochester, NY). The relative amount of 125I-Protein A bound to the immunoreactive polypeptides was quantitated by cutting out the labelled bands and counting in a gamma counter. Nonspecific binding was corrected for by subtracting the counts associated with a similar-sized piece of nitrocellulose excised from a blank portion of the same lane.

**RESULTS**

Actin, tropomodulin (Tmod), and tropomyosin (TM) were localized in spread preparations of isolated erythrocyte membrane skeletons by immunogold labelling followed by negative staining to visualize the structural features of the membrane skeletons. The morphological features of these immunogold-labelled membrane skeletons (long, thin spectrin filaments attached in a hexagonal pattern to short, thicker filaments at central junctions) are clearly visible (Fig. 1). Immunogold labelling for actin is associated with the short filaments at the central junctions (Fig. 1A), as is immunogold labelling for Tmod (Fig. 1B) and TM (Fig. 1C). Very little labelling with any of the three antibodies is associated with the spectrin filaments or with other sites in these membrane skeleton preparations. Furthermore, grids labelled with preimmune rabbit IgG are devoid of labelling, with the exception that gold particles occasionally adhered nonspecifically to the carbon substrate (Fig. 1D).

To quantitate the specificity of immunogold labelling for each of the three antibodies, the percentage of labelled central junctional complexes was compared to the percentage of labelled spectrin filaments (Table 1). The fraction of junctional complexes labelled by each of the three antibodies varied considerably: anti-Tmod antibodies labelled the largest fraction of junctions (32±3%), followed next by anti-β-actin antibodies (15±2%) and finally by anti-TM antibodies (9±2%). Statistical analysis of the results indicate that the fraction of junctional complexes labelled by antibodies to Tmod, TM, and actin is significantly different (P<0.05) from the fraction of spectrin filaments that were labelled by each antibody (3±0.6%, 3±1% and 2±1%, respectively). The fractions of junctional complexes or spectrin filaments labelled by preimmune rabbit IgG were both negligible (less than 1% in either case), again supporting the specificity of our antibodies.

![Fig. 2.](image) Comparison of the amounts of tropomyosin, tropomodulin and actin associated with the membrane skeleton before and after incubation in low ionic strength buffer in preparation for spreading. Equivalent amounts of membrane skeletons (lane 1), or supernatant (lane 2) and pellet (lane 3) obtained after dilution and centrifugation were electrophoresed on an SDS-gel and either stained with Coomassie Blue (A) or transferred to nitrocellulose and labelled with the indicated antibodies followed by 125I-Protein A (B). Membrane skeletons were prepared as described in Materials and Methods and diluted with 9 vol of 0.1 m NaPi, 0.5 mM DTT, pH 7.4. After incubation for 90 minutes on ice, skeletons were collected by centrifugation at 100,000 g in a Beckman 70Ti rotor, and resuspended to the original volume of the undiluted skeletons. Samples of the membrane skeleton and the resuspended pellet were prepared for electrophoresis by addition of 1/4 vol. 5x SDS-gel sample buffer. A sample of the supernatant was prepared by precipitation with ice-cold 10% trichloroacetic acid (Fowler and Bennett, 1984a) followed by solubilization in SDS-gel sample buffer in a volume equivalent to that of the resuspended pellet. Antibodies to TM (M, 27,000 and 29,000), Tmod (M, 42,000) and β-actin (M, 42,000) label the expected polypeptides (Fowler and Bennett, 1984a; Fowler, 1990). Anti-Tmod antibodies also label variably a minor Mr ~50,000 polypeptide (compare lanes 1 and 3; also see Fowler, 1990; Fowler et al., 1993) This polypeptide may be a Tmod-related protein present in low amounts in erythrocytes.
To determine if the relatively low efficiencies of labelling for Tmod, TM and actin were due to extraction of these proteins during preparation of the spread membrane skeletons, samples of the isolated skeletons were prepared under the same conditions as for microscopy, electrophoresed on SDS-gels, transferred to nitrocellulose membranes and probed with primary antibodies to Tmod, TM, or actin followed by detection with 125I-Protein A (Fig. 2). This experiment showed that all of the actin, approximately 90% of the Tmod, and 80% of the TM were retained during preparation of skeletons. Therefore, the low efficiencies of labelling for actin, Tmod and TM (approximately 15%, 32% and 9% of central junctions, respectively) may be primarily due to the inaccessibility of antigenic sites in the spread membrane skeletons and/or destruction by the fixation procedures.

High magnification views of negatively stained central junctions that were immunogold labelled with antibodies to Tmod, TM and actin are presented in Fig. 3. Although labelling for Tmod appears to preferentially occur at a single end of the actin filaments in these images (Fig. 3A), this was not observed when 5 nM gold-conjugated secondary antibody was used (data not shown; cf. Fig. 1B). Furthermore, the combination of primary and secondary antibodies could easily result in the gold particle being located as far as 30 nm away from the actual primary antibody binding site. Thus, this immunolocalization technique does not provide sufficient resolution to distinguish labelling at the end vs the middle of the 33 nm actin filaments.

Since negative staining of samples involves potentially disruptive air drying, isolated skeletons were also visualized by the more gentle method of quick-freeze, deep-etch, rotary replication (QFDERR). Skeletons visualized by this technique display many of the same features of their negatively stained counterparts, including easily distinguishable central junctional complexes (cf. Ursitti and Wade, 1993). Fig. 4 is a representative image of high resolution platinum replicas of skeletons that were immunogold-labelled with antibodies to Tmod. These images demonstrated a labelling pattern similar to that seen in the negatively stained samples (Fig. 1B), where gold labelling was mostly found at the central junctional complexes containing the actin filaments.

**DISCUSSION**

In this report, we have specifically localized erythrocyte Tmod, TM and actin at the ultrastructural level in isolated spread membrane skeletons. Direct immunolocalization of actin to the central junctions confirms previous indirect evidence that the short 33 nm filaments at these junctions are short actin filaments. Furthermore, immunolocalization of TM and Tmod to these same sites confirms predictions from relative stoichiometries and analysis of biochemical interactions that TM and Tmod are associated with the short erythrocyte actin filaments.

The percentage of labelled actin filament sites (central junctions) obtained in our experiments with antibodies to Tmod (32%) and β-actin (15%) compares favorably with the labelling efficiency obtained by others with antibodies to protein 4.1 (25%), dematin (18%) and adducin (19%) (Derick et al., 1992). Furthermore, statistical analysis indicates that the antibodies are specifically recognizing their antigens in the central junctional complexes (see Table 1). The relatively

<table>
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<tr>
<th>Primary antibody</th>
<th>% Central junctions labeled</th>
<th>% Spectrin filaments labeled</th>
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<tr>
<td>Tropomodulin</td>
<td>32±3*</td>
<td>34±0.6</td>
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<tr>
<td>(15, 1267)†</td>
<td>(15, 2086)</td>
<td></td>
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<tr>
<td>Tropomyosin</td>
<td>9±2</td>
<td>2±1</td>
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<tr>
<td>(8, 610)</td>
<td>(8, 1097)</td>
<td></td>
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<tr>
<td>β-Actin</td>
<td>15±2</td>
<td>3±1</td>
</tr>
<tr>
<td>(8, 729)</td>
<td>(8, 1062)</td>
<td></td>
</tr>
<tr>
<td>Preimmune</td>
<td>0.6±0.4</td>
<td>0.2±0.4</td>
</tr>
<tr>
<td>(5, 228)</td>
<td>(5, 394)</td>
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*Numbers are reported as percentages of total sites labelled±s.d.
†Number of micrographs analyzed for a particular antibody preparation, number of actual sites scored.

Fig. 3. High magnification views of central junctions after immunogold labelling with actin, tropomyosin and tropomodulin antibodies. Isolated skeletons were incubated with primary antibodies for (A) tropomodulin, (B) tropomyosin and (C) β-actin, followed by incubation with secondary antibodies adsorbed to either 5 nm (for actin and tropomyosin) or 10 nm (for tropomodulin) gold particles. Arrows indicate labelled central junctions.
lower efficiency observed for TM labelling in our experiments (only 9% of junctions labelled) could be due to partial loss of TM from membrane skeletons during preparation and labelling. About 20% of the TM was extracted from membranes during the last step in preparation of spread membrane skeletons (see Fig. 2). This step involved dilution into a large volume of low ionic strength buffer in the absence of magnesium, conditions that have been reported previously to extract tropomyosin from the membrane (Fowler and Bennett, 1984a). Similarly, additional TM could have been lost during the subsequent antibody incubations in the absence of magnesium. Although inclusion of millimolar magnesium in the antibody incubation buffers appeared to increase gold labelling for TM in these skeletons (data not shown), magnesium could not be included routinely in the antibody incubation solutions since it inhibited the ability of the antibodies to spread. This made it difficult to positively identify the structural elements in the skeletons and to quantitate the labelled structures (cf. Ursitti and Wade, 1993).

The relatively high efficiency of labelling with Tmod antibodies (32%) in comparison to labelling of all other actin-associated proteins in the erythrocyte membrane skeleton (see above) could be explained partly by the location of Tmod at the extreme ends of the short actin filaments, leading to an enhanced availability of Tmod sites for the antibodies. Although the resolution from immunogold labelling techniques was not sufficient to directly address this point, Tmod is likely to be associated with the pointed (slow growing) ends of the short actin filaments based on our recent immunofluorescence localization of Tmod to the pointed ends of actin filaments in skeletal muscle (Fowler et al., 1993) and the ability of Tmod to cap actin filament pointed ends in vitro (A. Weber, C. Pennise, G. Babcock and V. Fowler, unpublished observations).

Association of Tmod with the pointed ends of the short actin filaments in the erythrocyte membrane skeleton could account for previous observations that the pointed ends of the actin filaments are capped and unavailable for monomer addition and filament elongation in situ (Byers and Branton, 1985; Pinder et al., 1986). Thus, our immunolocalization of TM and Tmod to the short actin filaments supports the possibility that Tmod and TM could function together to regulate the length and stability of the erythrocyte actin filaments. Restriction of actin filament length is one possible mechanism to regulate the number of spectrin molecules attached to the actin filaments at the central junctions. This is expected to be critical for the symmetry, long range organization, and functional properties of the membrane skeleton (Liu et al., 1990).

The authors thank Dr J. C. Bulinski (Columbia University College of Physicians and Surgeons, New York, NY), Dr I. M. Herman (Tufts University, Boston, MA), and Dr D. W. Speicher (The Wistar Institute, Philadelphia, PA) for kindly providing the actin antibodies used in this study. We especially thank Dr J. B. Wade (University of Maryland School of Medicine, Baltimore, MD), in whose laboratory the electron microscopy work was performed, for his helpfulness and generosity. We also thank Marcus Shagogue for assistance with photography, and Carol Gregorio for editorial suggestions on the manuscript. This work was supported by a grant from the NSF, DCEB-8819105 (J.A.U.) and from the NIH, GM34225 (V.M.F.).

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(Received 7 January 1994 - Accepted 15 February 1994)