Erythrocyte Tropomodulin Isoforms with and without the N-terminal Actin-binding Domain*

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Erythrocyte tropomodulin (E-Tmod or Tmod1) of 41 kDa is a tropomyosin (TM)-binding protein that caps the slow-growing end of the actin filaments. Its N-terminal half is flexible, whereas the C-terminal half has a single domain structure. E-Tmod/TM5 complex may function as a “molecular ruler” generating actin protofilaments of ~37 nm. Here we report the discovery of a short isoform of 29 kDa that lacks the N-terminal actin-binding domain (N-ABD) but retains the C-terminal actin-binding domain (C-ABD). E-Tmod29 can be generated by alternative splicing from an upstream promoter or by multiple transcriptional start sites from a downstream promoter. Promoter switching leads to a surge of E-Tmod41 in reticulocytes, which degrades quickly in the cytosol. We expressed recombinant isoforms in Escherichia coli and tested their binding toward TM5, G-actin, and F-actin. Solid-phase binding assays show that, without the N-terminal 102 residues, E-Tmod29 binds to TM5 or G-actin more strongly than E-Tmod41 does, but barely binds to F-actin after TM5 binding. Differential bindings explain the distinct localizations of E-Tmod29 in the cytosol and E-Tmod41 on the membrane. Sequential bindings and immunofluorescent staining further suggest that 1) TM5 binding to E-Tmod41 may open up the flexible N-terminal half, exposing N-ABD and unblocking C-ABD; 2) N-ABD binds to F-actin and C-ABD binds to G-actin; and 3) F-actin binding to N-ABD may prevent G-actin from binding to C-ABD. E-Tmod29 may thus modulate the availability of TM5 and G-actin for E-Tmod41 to construct the protofilament-based membrane skeletal network for circulating erythrocytes.

Erythrocytes are responsible for the transport of O2 and CO2 in tissues throughout the body. Their cell membranes have a thin layer of protein skeleton under the lipid bilayer. The viscoelasticity and durability of this protein skeleton allows erythrocytes to circulate for 120 days in humans and 60 days in mice. These membrane skeletal proteins are differentially expressed during erythroid differentiation, and the complex transcellular cytoskeleton is remodeled into a membrane skeleton during reticulocyte maturation (1, 2).

The membrane skeletal network consists of a large number of basic repeating units interconnected to each other. Each unit contains two kinds of major complexes, a junctional complex (JC) and suspension complex (SC), located in the center and up to six in the periphery, respectively (Fig. 1). A JC includes actin, tropomyosin (TM), erythrocyte tropomodulin (E-Tmod), protein 4.1R, adducin (3), protein 4.9 (dematin), and p55 (for review see Refs. 3 and 4), with six spectrin heterodimers (Sp) radiating from the center. Associated near the distal end of each Sp is an SC. An SC consists of several proteins, including anion exchanger (band 3), ankyrin, and protein 4.2, which suspends the skeletal network to the lipid bilayer (for review see Refs. 5 and 6).

Some of these membrane skeletal proteins are encoded by genes known to have alternative splicing, alternative promoters, multiple transcriptional start sites, and/or multiple polyadenylation sites (7–14). Indeed, some in JC are cell type-specific isoforms generated by one or more of the above mechanisms. For example, exon 16 of protein 4.1R, which encodes the 8-kDa spectrin/actin-binding domain (ABD), is alternatively spliced (15, 16). β spectrin, which has an ABD, is generated by the differential splicing of its exon 32 (9). Interestingly, α spectrin, which has no ABD, is synthesized three times more than β spectrin, but is quickly degraded in reticulocytes if not incorporated into the membrane skeleton (17). Thus, coordinated expression between ABD-containing and ABD-lacking proteins and isoforms is crucial to the construction of the network.

Additionally, 41-kDa E-Tmod (18) is also an ABD-containing protein (19). It caps the slow-growing end (pointed or minus end) (20, 21) of TM-coated actin filaments. First isolated as a TM-binding protein (22), it binds to the N terminus of TM (23, 24), forming a complex with two shorter TM isoforms 5 or 5b (~35 nm) (25), encoded by the γ and α genes, respectively. Such complexes may function as a “molecular ruler” (a T ruler with a cap), metering off long actin filaments to short protofilaments of ~37 nm (25, 26).

In our previous study on “protofilament and hexagon” (26), we illustrated that the length of the protofilament dictates the hexagonal topology of the skeletal network. We showed that a protofilament reinforced by TM (one in each groove) and the availability of TM5 and G-actin for E-Tmod41 to construct the protofilament-based membrane skeletal network for circulating erythrocytes.

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The abbreviations used are: JC, junctional complex; SC, suspension complex; TM, tropomyosin; E-Tmod, erythrocyte tropomodulin; Sp, spectrin heterodimer; ABBD, actin-binding domain; N-ABD, N-terminal ABD; C-ABD, C-terminal ABD; TBS, TM5 binding site; RACE, rapid amplification of cDNA ends; MEL, murine erythroleukemia; pAb, polyclonal antibody; DB, destruction box.
The N-terminal actin-binding domain (N-ABD) contains an actin-binding activity was found in two distinct regions (36). An N-terminal end of TM exhibit drastic structural changes upon binding to each other (35). The regions of E-Tmod involved in actin and TM bindings have been investigated in different species. In chickens the N-terminal half of E-Tmod and E-Tmod isoforms derived from the E-Tmod gene have been characterized (33). Each has nine coding exons (E1–9) and further upstream, one 5′ non-coding exon (E0). Both species have an upstream promoter 5′ to E0 and a downstream promoter 5′ to E1. The capping of actin filament at the slow growing end by E-Tmod is essential: an embryonic lethality was revealed when the E-Tmod gene is disrupted at either E1 (40) or E2 (41). The homozygous E1 KO embryos die approximately at embryonic day 10, exhibiting a non-contractile heart tube, accumulation of mechanically weakened primitive erythroid cells, and failure of primary capillary plexuses to remodel into vitelline vessels (40). In this study, we report the discovery of a new E-Tmod isoform of 29 kDa in mice that has a truncated N terminus. We illustrated how isoforms with and without N-ABD are generated. In addition, we identified their distinct cellular distributions, verified the functionality of both promoters, expressed recombinant isoforms, and tested their binding activities toward TM5, G-actin, and F-actin. Based on these results, we formulated a model to show how N- and C-terminal ABDs may function and participate in the formation of E-Tmod/TM/actin complex as well as how these two isoforms may coordinate during the construction of the membrane skeletal network for erythrocytes.

**EXPERIMENTAL PROCEDURES**

**Animals, Tissues, and Cells**—Adult mice of C57BL wild type were used to collect erythrocytes, aortae, uteri, and hearts. Erythrocytes were lysed in lysis buffer (6 mM Na2HPO4, 1.5 mM NaH2PO4, 1 mM EDTA, 20 mg/liter PMSF, 2 mg/liter pepstatin) to obtain whole cell lysate. Ghost membranes were isolated by density gradient centrifugation. Erythrocytes were lysed in hypotonic solution.

**FIGURE 1. Schematic drawings of the erythrocyte membrane skeleton.**

A, an expanded “spoked” hexagonal network, based on electron micrographs (56) of erythrocytes in hypotonic solution; B, a junctional complex (JC), showing a protofilament of ~37 nm associated two TM, an E-Tmod and six Sp; and C, E-Tmod/TM complex as a molecular ruler for the protofilament. SC, suspension complex; TM5/5b, tropomyosin isoform 5 and 5b homo- or heterodimer (modified from Ref. 26).

Capped by E-Tmod may function as a mechanical axis to anchor 3 (top, middle, and bottom) pairs of Sp, forming a 6-armed structure. There, we reported the first three-dimensional model for a JC and how each pair of G-actin may be wrapped around by a split α and β spectrin (Sp) and stabilized by 4.1R, forming a basic repeating unit (26). This model allows the three-dimensional nano-mechanics of a JC or a basic unit to be simulated in terms of the attitude (pitch, yaw, and roll angles) of the protofilament and the tension of each radiating Sp (in pN) during equibiaxial and anisotropic deformations (27, 28).

E-Tmod is also present in non-erythroid cells and tissues. In muscular cells, it is located at the free end of thin filaments in myofibrils (20) and costameres in the skeletal muscle (29). It is also expressed in lens fiber cells in the eye (30) and neurons (31) in the CNS (32). The Tmod family contains several homologous genes across several species, including vertebrates and non-vertebrates (for summary, see Table 1 in Ref. 33). Using antibodies, several immunoreactive peptides of various sizes have been detected in a number of cell types of different species (34). But no isoforms derived from the E-Tmod gene have been identified.

E-Tmod is highly conserved among species (including human, mouse, rat, and chicken). The percentage of identity between mouse and several other species in each exon has been tabulated (33). In particular, no insertion or deletion was found in exon 6 (E6) and exon 7 (E7) within the entire family of more than 15 members, including those more remote, different-sized homologs. CD and differential scanning calorimetry have been used to study chicken E-Tmod. The N-terminal half lacks a definite tertiary structure in solution, whereas the C-terminal half (resides 160–344) represents a single, cooperatively melting domain. Furthermore, the N-terminal half of E-Tmod and the N-terminal end of TM exhibit drastic structural changes upon binding to each other (35).

The regions of E-Tmod involved in actin and TM bindings have been investigated in different species. In chickens the actin-binding activity was found in two distinct regions (36). An N-terminal actin-binding domain (N-ABD) contains an actin binding site involving residues 1–92 in chickens (37) or residues 71–104 in humans (38, 39). Specifically, a L71D mutation destroyed the capping function in chickens (39). There is another ABD located in the C-terminal half (36) (chicken). Interestingly, a TM5 binding site (TBS) is positioned immediately downstream from the N-terminal actin binding site. In humans, TBS was mapped to residues 105–127 with LE/E (116–117/118) being the critical residues (38). Furthermore, it has been shown that E-Tmod does not bind to G-actin when F-actin is present in the solution (21). It is not clear, however, whether the N- or C-terminal actin binding site is for G-actin or F-actin.

Both human and mouse E-Tmod (Tmod1) genes have been characterized (33). Each has nine coding exons (E1–9) and further upstream, one 5′ non-coding exon (E0). Both species have an upstream promoter 5′ to E0 and a downstream promoter 5′ to E1. The capping of actin filaments at the slow growing end by E-Tmod is essential: an embryonic lethality was revealed when the E-Tmod gene is disrupted at either E1 (40) or E2 (41). The homozygous E1 KO embryos die approximately at embryonic day 10, exhibiting a non-contractile heart tube, accumulation of mechanically weakened primitive erythroid cells, and failure of primary capillary plexuses to remodel into vitelline vessels (40).
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...dures are approved by the Animal Care Program at the University of California, San Diego.

**Western Blot Analysis**—Ten micrograms of ghost membrane or 100 µg of total proteins from other tissues were mixed with 2 x SDS loading buffer (containing 200 mM DTT) and separated by SDS-PAGE on a 10% gel (pH 8.8). Protein profiles were transferred onto a nitrocellulose membrane and incubated with mAb 204 (1:150) or pAb 2644 (1:100) (23). The former was followed by an HRP (horseradish peroxidase)-conjugated goat-anti-mouse IgG incubation (1:3000) and chemiluminescent detection; the latter by HRP-conjugated goat-anti-rabbit IgG secondary antibody (1:1500) and colorimetric development.

**RNA Isolation, RT-PCR, and cDNA Cloning and Sequencing**—Total RNA was isolated from the aorta, heart, uterus, and yolk sac using TRIzol Reagent (Invitrogen, Carlsbad, CA). Reticulocyte RNA was extracted from reticulocyte-enriched blood using partial lysis combined with TRIzol (43). 1 µg of RNA was reverse-transcribed using SuperScript III reverse transcriptase and Oligo(dT) 12–18 primers (Invitrogen). 2 µl of cDNA was amplified using a forward primer, 5'-CGCCAGGGAGACCTGCCCAAG-3', and a reverse primer, 5'-GACACGCGATCATCGGTGTAAGAC-3', located in E0 and E9, respectively. Amplification (35 cycles) was performed under the following conditions: denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 2 min at 68°C. DNA fragments were analyzed by 0.9% agarose gel electrophoresis. The PCR products were then inserted into pEGFP-N1 vector (Invitrogen) with a N-terminal 6× His tag. The plasmids (pET-E1–9 and pET-E0/3–9) were transformed into BL21 Star™ (DE3)-competent cells. The recombinant proteins were induced to express by isopropyl β-d-thiogalactopyranoside (0.5 mM). The bacterial cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100) followed by sonication. The supernatant was collected by centrifuging at 4,000 × g for 30 min. The recombinant proteins in the supernatant were purified at 4°C with nickel column (Sigma).

**Solid Phase Binding Assay**—The procedure was based on Sung and Lin (23). Briefly, E-Tmod isoforms (1 µg) were separated by 10% SDS-PAGE, transblotted onto a nitrocellulose membrane, and overlaid with recombinant human (h) TM5, G-actin (extracted from rabbit skeletal muscle, Cytoskeleton Inc., Denver, CO), or F-actin. The transblot was incubated with either a mouse anti-TM5 mAb CG3 (IgM, 1:1000, epitope mapped to residues 29–44 and kindly provided by Jim Lin) or mouse anti-human actin mAb AC-40 (1:500) to detect the EGFP fusion proteins.

**Quantification of the Binding Signals**—We measured the densities of the bands with ImagePro software and divided the values by the amount of isoform protein used to obtain the pixels/microgram of protein values. The ratio of binding signals between the two isoforms was then calculated. To avoid the variations caused by the molecular weights, we further calculated their pixel/mole of protein values based on their molecular weights, and the final signal ratio was again obtained.

**Double Immunofluorescent Staining**—Erythroblasts from yolk sacs, reticulocyte-enriched blood, and erythrocytes were fixed in 4% paraformaldehyde and washed three times in the mouse RBC solution. The cells were incubated in the blocking solution (2% BSA and 0.1% Triton X-100 in the mouse RBC solution) for 30 min and then incubated with mAb 204 (1:100) at 4°C for 2 h. After washing, the cells were incubated with goat-anti-mouse IgG-FITC (1:250) at 4°C for 45 min. F-actin was then stained by incubating the cells with rhodamine-phalloidin (5 units, Invitrogen). After washing, cells were re-suspended in 2% gelatin and mounted on coverslips. The fluorescent images were taken with a confocal micro-
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To test if $M_r$ 58,000 is a dimer of $M_r$ 29,000 (which may be a short isoform of E-Tmod) we omitted DTT in one uterus sample (u−), whereas all other samples, including u, were treated with 100 mM DTT. Without DTT to break disulfide bonds, $M_r$ 58,000 was found to increase, whereas $M_r$ 29,000 decreased in u−. Further, by increasing DTT to 200 mM we converted all $M_r$ 58,000 to $M_r$ 29,000 in uterus samples (data not shown). Thus, $M_r$ 29,000 and $M_r$ 58,000 are indeed mono- and dimeric forms of the same molecule.

The relative abundance of $M_r$ 41,000 and $M_r$ 29,000 is different among tissues. In the heart, $M_r$ 41,000 is the predominant species while $M_r$ 29,000 is the principal in the aorta and the uterus (smooth muscles). The ratios between the two species ($M_r$ 29,000/$M_r$ 41,000) in aorta, uterus, and heart are ~8, 35, and 0.6, respectively (Fig. 2B). In erythrocytes $M_r$ 41,000 is confirmed to be the sole species in the ghost membrane (g). As to the newly discovered $M_r$ 29,000 and its dimer, it is the main (or only) species in the cytosol (see e). $M_r$ 29,000 (in the cytosol) may outnumber $M_r$ 41,000 (in the membrane) on the per cell basis, because the latter was not detectable in the whole cell sample (see e).

Alternative Splicing—We tested for the possibility that $M_r$ 29,000 is a shorter E-Tmod isoform. Because anti-E2 pAb only detects the larger $M_r$ 41,000 species and anti-E6/7 mAb detects both (Fig. 2, A and B), $M_r$ 29,000 may be a splice variant in which E2 is spliced out. Therefore, RT-PCR with gene-specific primers derived from E0 and E9 was performed to reveal the inclusion or exclusion of E2 (Fig. 2C). The cDNA prepared from reticulocytes (re), which still contain some mRNA, was used as a template. cDNAs were also prepared from MEL cells (m), aorta (a), heart (h), and uterus (u).

In the end, three major RT-PCR fragments were revealed (Fig. 2C). These three fragments were then individually isolated, cloned, and sequenced. The largest band was the cDNA of the full-length E-Tmod transcript, from E0 to E9 (called the E0–9 transcript). Such a transcript would encode a full-length E-Tmod of 359 residues with a calculated molecular mass of 40.5 kDa (referred to as E-Tmod41), as previously reported (18, 32, 33). The smallest band starts from E0, skips both E1 and E2, and extends from E3 to E9 (called the E0–3/9 transcript). Such a transcript would use an in-frame ATG in E3 as the initiation codon. Thus, an E0/3–9 transcript may encode a truncated isoform missing the N-terminal 102 residues. It would have 293 residues with a calculated molecular mass of 28.5 kDa (referred to as E-Tmod29). The exon utilizations for these two major isoforms are illustrated in panel D of Fig. 2.

The middle band (**) in Fig. 2C represents a transcript that skips E2 only but has a stop codon TGA in E3. This transcript would produce a very short peptide (57 residues) with no similarity with E-Tmod (starting from residue 41). It would not be detected by either E-Tmod-specific antibodies and is not further pursued here.

Promoter Switching—We previously reported the presence of two alternative promoters, upstream from E1 and E0, respectively (33). To find out their relative usage in isoform expression, 5′ RACE was performed using a reverse primer derived from E3 (shared by both isoforms). The mRNAs were extracted from nucleated erythroblasts from yolk sacs and reticulocytes...
expression of EGFP driven by these two promoter sequences was detected. MEL cells were used because they should express transcription factors to drive these promoters. Based on flow cytometry, promoter $P_{E1}$ appeared to be stronger than promoter $P_{E0}$ under this condition (Fig. 3F).

**Multiple Transcriptional Start Sites**—Detailed sequence analysis on the 5′-RACE CDNA fragments revealed multiple transcriptional start sites (labeled as solid circles in Fig. 3, D and E), like other TATA-less promoters (45, 46). As illustrated in Fig. 3E, reticulocyte transcripts starting anywhere within E0 and retain E1/2 would be translated into E-Tmod41. Interestingly, a small number of transcripts starting within E1 before the ATG initiation codon would also generate E-Tmod41. Therefore, the long isoform may be generated from both promoters, with the majority being transcribed from the upstream promoter.

In erythroblasts (Fig. 3D), many transcripts start within E1 but after ATG. They would use the next available ATG in E3 and generate E-Tmod29. Interestingly, this would be exactly the same product generated from the alternatively spliced transcripts that start from E0 but skip E1/2 (transcript E0/3–9). Consequently there are two mechanisms by which E-Tmod29 may be generated: 1) alternative splicing using the upstream $P_{E0}$ promoter (Fig. 2D) and 2) multiple transcriptional start sites 3′ to the first ATG using the downstream $P_{E1}$ promoter (Fig. 3D).

The Fate of Two Isoforms—In erythroblasts, because more transcripts start from E1 after the first ATG, more E-Tmod29 is expected. Indeed, a large amount of E-Tmod29 was detected in the E9.5 yolk sac (Fig. 3C). In reticulocytes, essentially all transcripts start from E0 (and contain E1/2), based on 5′-RACE (Fig. 3A) and RT-PCR (Fig. 2C), therefore, the main proteins synthesized would be E-Tmod41. But mature erythrocytes had no detectable E-Tmod41 in the cytoplasm. E-Tmod41 was only detected in the membrane and was outnumbered by E-Tmod29 in the cytosol (Fig. 2B, lane e). To investigate whether E-Tmod41 exists in the cytosol of reticulocytes before becoming membrane-bound in erythrocytes, we isolated their cytosolic fractions. Western blot analysis indeed detected E-Tmod41 in the cytosolic fraction of reticulocytes (Fig. 4A, retic) but no longer in that of mature erythrocytes (RBC) (Fig. 2B, lane e). To investigate whether E-Tmod41 exists in the cytosol of reticulocytes before becoming membrane-bound in erythrocytes, we isolated their cytosolic fractions. Western blot analysis indeed detected E-Tmod41 in the cytosolic fraction of reticulocytes (Fig. 4A, retic) but no longer in that of mature erythrocytes (RBC). Results suggest: 1) E-Tmod41 may have a much shorter half-life in the cytoplasm and/or 2) there may be a preferential incorporation of E-Tmod41 onto the membrane during reticulocyte maturation (~2 days).

To test the half-life of these two isoforms, their cDNAs were fused with EGFP. Plasmids containing E1–9/EGFP and E0/3–9/EGFP sequences, which were under the control of a CMV promoter, were transiently transfected into C2C12 myoblasts. Both fusion proteins were expressed by 24 h as examined by fluorescence microscopy (data not shown). By 72 h, however, E-Tmod41/EGFP had disappeared while E-Tmod29/EGFP remained consistent with the findings revealed by Western blot analysis (Fig. 4B). The results clearly indicate a quicker degradation of E-Tmod41 than E-Tmod29. C2C12 myoblasts were chosen for transfection, because they have lower and undetectable level of endogenous E-Tmod expression.

**Two Recombinant Isoforms in E. coli**—To understand their binding properties and cellular localization, cDNAs of E0–9 enriched peripheral blood from adult mice. Gel electrophoresis revealed two major 5′-RACE fragments from embryonic erythroblasts (E.B.) starting from E0 and E1, respectively, but only one major fragment from adult reticulocytes (retic) starting from E0 (Fig. 3A). Several clones were sequenced to reveal their 5′-ends. In E.B. more 5′-RACE fragments started from E1 than E0 (Fig. 3A). In retic essentially all of them start from E0 (and without splicing, see below). This result clearly indicates a promoter switching (from the downstream promoter $P_{E1}$ to the upstream promoter $P_{E0}$) to up-regulate the expression of E-Tmod41 at the reticulocyte stage.

To confirm that shorter transcripts in erythroblasts are not alternatively spliced E0/3–9, all fragments were digested with an E2-specific restriction enzyme BglII (Fig. 3B). The fact that they were digested into smaller fragments indicates that it is not the E2 splicing, but indeed the use of the downstream promoter that results in shorter transcripts.

To demonstrate that genomic sequences upstream from E0 and E1 indeed serve as promoter sequences, they were PCR-cloned and inserted into a plasmid upstream from the EGFP cDNA sequence. After a stable transfection in MEL cells the
and E0/3–9 were cloned by RT-PCR using mouse reticulocytes and expressed in E. coli. SDS-PAGE (Fig. 4D) revealed that the purified recombinant E0–9 protein plus a His tag (called r41) is ~50 kDa, and the purified recombinant E0/3–9 protein plus a His tag (called r29) is ~36 kDa. Specifically, we studied their binding toward TM5, G-actin, and F-actin. In the solid-phase binding assay, r41 and r29 were first separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and overlaid by the binding proteins (Fig. 5).

**Binding to TM5**—Recombinant TM5 was applied and bound TM5 was detected by anti-TM5 mAb CG3 (epitope mapped to residues 29–44 on TM5). Fig. 5A shows that both r41 and r29 could bind to TM5, but r29 appeared to bind ~133% more than r41 did (see “Experimental Procedures”). Results suggest that, without the N-terminal 102 residues, the TM5 binding site (TBS or T) immediately downstream (within residues 105–127) (38) may become more accessible (as illustrated in Fig. 5B).

**Binding to G-actin**—G-actin was applied, and bound G-actin was detected by anti-actin mAb AC-40. Fig. 5C shows that both could bind to G-actin. Because r29 has only one ABD (the C-terminal ABD) it must be a G-actin-binding domain. The binding experiment showed that r29 binds ~26% more G-actin than r41. Results suggest that without N-terminal 102 residues, the C-terminal, G-actin binding site (G) may become more accessible in r29 (Fig. 5D). It is reasonable that, because the N-terminal half of E-Tmod41 is flexible (35), it may partially block the C-terminal G-actin binding site.

**Binding to TM5 followed by G-actin**—TM5 was applied before G-actin, allowing isofoms to bind to TM5 before interacting with G-actin. The bound G-actin was then detected. Fig. 5E shows that both could bind to TM5/G-actin, but the binding of r29 was ~84% that of r41. Results suggest that binding of the rod-like TM5 to r41 at TBS in the mid-section between the N- and C-terminal halves may improve its ability to bind to G-actin, perhaps, by opening up the two halves (see Fig. 4E) and unblocking the C-terminal G-ABD (as illustrated in Fig. 5F). An earlier study, reporting that upon binding to TM the N-terminal half of E-Tmod exhibited drastic structural changes (35), supports this possibility.

**Binding to TM5 followed by F-actin**—Recombinant isoforms on the nitrocellulose membrane were to bind to TM5 first (to induce the N-terminal conformational changes) before interacting with F-actin. Fig. 5G shows that r41 now binds to F-actin strongly while r29 barely does. Results reveal that the rod-like TM5 binding to r41 at TBS between the N- and C-terminal domains makes N-ABD more accessible to F-actin. As illustrated in Fig. 5H, this may be achieved by opening up the N-terminal half and exposing N-ABD for F-actin. The bound TM5 may further assist the binding or formation of F-actin on

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4 W. Yao, T. Green, and L. A. Sung, unpublished results.
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In the reticulocyte-enriched blood sample (middle row), which was collected 2 days after the 5-day phenylhydrazine treatment, there were a large number of highly fluorescent “green” cells, not seen in untreated blood samples. The strong cytosolic signals may reflect the surge of E-Tmod41 in reticulocytes. The intensity may depend on the age or the time (up to 2 days) each reticulocyte had been released into circulation: the older they are, the more E-Tmod41 may be degraded in the cytosol or incorporated into the membrane. The two cells in Fig. 6A (middle row) that no longer possessed any detectable green signals may be mature erythrocytes. Membrane signals on mature erythrocytes were not visible in this image, because the detection sensitivity had been drastically tuned down to accommodate the overwhelming signals in reticulocytes. The F-actin had membrane enrichment pattern within all of the cells. In erythroblasts (top row), both E-Tmod41 and F-actin had signal concentrations in the perinuclear area with no obvious membrane enrichment.

The Coordination between Two Isoforms—Based on what we have discovered, a model (Fig. 6C) illustrates how these two isoforms may coordinate in the construction of the membrane skeleton. In this study, we demonstrate that the lack of the N-terminal 102 residues diminishes the F-actin binding but greatly enhances the binding to TM5 and G-actin. The abundant E-Tmod29 in the cytosol may thus hold a large reservoir of TM5 and G-actin and play an important role in modulating the availability of TM5 and G-actin for E-Tmod41.
The increased E-Tmod41 in reticulocytes may bind to the available TM5 and open up N-ABD and C-ABD. N-ABD would bind to F-actin or C-ABD to G-actin. TM5 in N-ABD should interact with F-actin or serve as a template to polymerize G-actin. When G-actin is polymerized into F-actin, only the terminal G-actin at the slow growing end (pointed or minus end) has the site available to interact with the N-terminal F-actin. This also implies that if G-actin is polymerized on E-Tmod/TM5 (the molecular ruler) into a protofilament, it should grow from the slow growing end to the fast growing end (Fig. 1B).

**DISCUSSION**

In this study we utilized two specific antibodies, one specific to the N terminus and one specific to the C terminus, to identify a new E-Tmod isoform that has a truncated N terminus. The utilization of these two antibodies specific for two distinct epitopes to E-Tmod also permitted us to discover the accessibility of the epitopes in the actin-bound and free states.

**The Accessibility of Epitope—** Using mAb 204, E-Tmod29 in the cytosol could be detected by Western blot analysis (Fig. 2) but not in confocal microscopy (Fig. 6). There are two major differences between these techniques: 1) all proteins are denatured (and perhaps partially renatured) in the Western blot analysis, but not in immunofluorescent staining and 2) all proteins are dissociated during electrophoresis, but not in situ staining. Because mAb 204 could detect E-Tmod41 in both techniques the issue is not denaturation but blockage of the epitope by associated protein(s). The critical question is which protein binds E-Tmod29 and likely blocks an epitope in the C-terminal E6/E7 region. Based on our findings, the top candidate is G-actin.

The epitope of mAb 204 has been mapped to include residues 212–249 within the region encoded by E6/E7 (Fig. 7B). E6 and E7 are the most highly conserved exons in E-Tmod (see Table 1 in Ref. 33). In fact, they are the only two exons without a single insertion or deletion (only substitution) of residues in the entire Tmod family, including vertebrates and invertebrates (33).
Therefore, E6/E7 encoded residues in the C-terminal structured domain must play an essential role for the structure and function of the family. Fig. 7B shows the partial sequence alignment of 18 members and identical residues are shaded, where a putative G-actin binding site may reside. Because only three residues (Tyr42, Glu250, and Met285) differ between human and mouse E-Tmod in this region, the epitope may include any of these three residues. This epitope is likely to be very close to a small cluster of residues (labeled by an asterisk) that are identical among all members and may be critical for G-actin binding. Thus, it is reasonable for E-Tmod29 to be unrecognized by mAb 204 when it is bound by G-actin.

The next critical question is why the membrane-bound E-Tmod41 was recognized when it is associated with F-actin in the immunostaining. This observation can be explained by the possibility that, when N-ABD is occupied by F-actin, C-ABD is no longer occupied by G-actin, thus making this epitope accessible. Because the binding sites for F-actin and G-actin are in close proximity (on the same side of E-Tmod41 when TM5 is bound), and G-actin (~42 kDa) is about the same size as E-Tmod41, it is reasonable that once F-actin (multiple G-actin) binds to E-Tmod41, G-actin binding site is no longer accessible by the globular G-actin. The epitope outside of the G-actin binding site, however, may still be available for the antigen binding site at the tip of mAb 204. A previous report that E-Tmod41 does not bind to G-actin when F-actin is present in the solution (21) also supports our findings.

**The Stability of E-Tmod41**—E-Tmod41 in the reticulocytic cytosol may degrade quickly. This is supported by 1) transfection experiments, which demonstrated that E-Tmod41/EGFP fusion proteins could only be transiently detected in C2C12 myoblasts (Fig. 4B) as well as a similar experiment using fibroblasts (48), and 2) two potential destruction boxes (DBs) found in N-ABD (Fig. 4C). Previous studies have shown that ubiquitin ligases are present in reticulocytes (49), which ubiquitinate the cytosolic α spectrin and induce its quick degradation (17). Interestingly, a spectrin becomes stable in the membrane skeletal network after association with β spectrin (17).

A partial alignment of E1/E2 encoded sequences among 18 Tmod family members (Fig. 7A) reveals a region without any insertion/deletion (i.e. gaps). This region is likely to be where the putative F-actin binding site may be located and the conservation implies that other members may also have an F-ABD segment. Anti-E2 pAb’s inaccessibility (to residues 70–84) when E-Tmod41 is bound with F-actin in striated muscles in situ suggests a nearby F-ABD. A cluster of identical residues shared by all members and labeled by an asterisk are likely to be critical for F-actin binding. A striking finding is that DB2 (residues 64–67 RXXL) is conserved in the entire family and included in this cluster. Thus, if E-Tmod41 is bound with F-actin, DB2 and nearby DB1 and DB3 may become inaccessible. The fate of E-Tmod41 may, therefore, depend on F-actin-bound and free states: E-Tmod41 is sensitive to degradation in the cytosol before binding to F-actin, but not after incorporation into the F-actin-based skeletal network. Thus, the quick rise and fall of E-Tmod41 in reticulocytes followed by the long lasting stability in the membrane may have a similar mechanism to α spectrin. In α spectrin, ubiquitination sites (residue Lys) have been identified in repeats 17 and 20 and part of 21 (50). Because α and β spectrin intertwine side-by-side in an anti-parallel fashion and the C terminus of α spectrin (repeats 20–21) interacts with the N terminus of β spectrin (51), their dimerization may block the ubiquitin-recognition sites in α spectrin (50). How α and β spectrin at its tail region may wrap around F-actin in a JC has been proposed (28).

**The Membrane Skeletal Network**—In this study, we report a new isoform of 29 kDa derived from the mouse E-Tmod gene, in addition to the known 41-kDa protein. We have demonstrated that the expression of these two isoforms may be regulated by some combination of 1) alternative splicing, 2) multiple transcriptional start sites, 3) alternative promoters, 4) promoter switch, 5) protein stability, and 6) final cellular destination during development and/or differentiation.

The membrane skeletal assembly and remodeling occur along the terminal differentiation and reticulocyte maturation. This is the time when the assembly of α and β spectrin into the membrane skeleton reaches the highest level of steady state (2). It is also the time that protein 4.1R (15, 52) is up-regulated to stabilize the actin-spectrin association. A surge of E-Tmod41 at this stage may also play a role in the assembly/remodeling process. It may facilitate the formation of molecular ruler (E-Tmod41/TM5) (Fig. 1C) and protofilament at the center of a JC (Fig. 1B) (26). By this process, E-Tmod41 becomes an integral part of the membrane skeletal network (Fig. 1A).

The surge of E-Tmod41 occurs in the background of abundant E-Tmod29. The latter may sequester or release TM5 and G-actin (Fig. 6C) in the cytosol and serve as a reservoir. Initially, when the complex of E-Tmod41/TM5/F-actin is formed, the actin filament may or may not be ~37 nm. However, given the stress and strain experienced in capillaries, which often have diameters smaller than that of erythrocytes, any long actin filaments would be fragmented or shortened to ~37 nm (10). This process may take ~2 days, because this is the typical time span that reticulocytes take to mature into erythrocytes (53). Both the ruler mechanism (TM5 with a length of ~33–35 nm) and the helicity mechanism (F-actin with an intrinsic helicity of ~37 nm) may contribute to the uniformity of the length (26). When a reinforced protofilament (E-Tmod/TM/F-actin) with such a defined length (consisting six pairs of G-actin) is formed, it may serve as the mechanical axis for three pairs of Sp to build a six-armed basic unit (Fig. 1B). As numerous basic repeating units join together, a seamless thin layer of the membrane skeletal network may be constructed to provide the durability and elasticity for circulating erythrocytes (Fig. 1A).

Irreplaceable E-Tmod41—We utilized recombinant isoforms to analyze their binding activity in vitro: our goal being to understand the biological function of E-Tmod29 in vivo in conjunction with E-Tmod41. When we created the E-Tmod knockout mouse model by target disruption of exon 1 (40), the embryos died at day 10. This E1 KO mouse model specifically knocked out E-Tmod41, leaving E-Tmod29 intact. Therefore, it is clear that E-Tmod29 is not able to replace E-Tmod41 for its biological function. This is because of the diminished F-actin binding of E-Tmod29 due to its lack of N-terminal F-ABD. Because E-Tmod KO is embryonically lethal, gene disruption approaches in vitro (54) or rescue of our E1 KO mouse model
may establish the precise functional role of E-Tmod in many important processes, including erythropoiesis. It may be harder to create cells with a lack or reduced level of E-Tmod29 without interfering E-Tmod41, because the short isoform is an integral part of the long isoform. Non-erythroid cells have various ratios between these two isoforms (Fig. 2B), so overexpressing E-Tmod29 in E-Tmod41 dominant cells or overexpressing E-Tmod41 in E-Tmod29 dominant cells may yield interesting results.

Implications in Non-erythroid Cells—Our discovery of the new isoform and the differential binding properties toward G-actin and F-actin between two isoforms has functional implications in both erythroid and non-erythroid cells. The implication to cardiomyocytes, for example, will be significant, because the major isoform E-Tmod41 deficiency is already known to cause embryonic heart failure and lethality (40, 41), and its dilated cardiomyopathy in adult mice (55). The implication to smooth muscle cells will also be significant, because we now revealed that E-Tmod29 is the dominant isoform in these cells known to organize very long actin filaments without defined length (or striations). Any system that regulates the balance between G-actin and F-actin should play an important role in actin-based network structures and cellular processes. Thus, the functional role of E-Tmod isoforms in erythroid and non-erythroid cells in vivo deserves further investigations.

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