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Tropomyosin-binding properties modulate competition between tropomodulin isoforms

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ABSTRACT

The formation and fine-tuning of cytoskeleton in cells are governed by proteins that influence actin filament dynamics. Tropomodulin (Tmod) regulates the length of actin filaments by capping the pointed ends in a tropomyosin (TM)-dependent manner. Tmod1, Tmod2 and Tmod3 are associated with the cytoskeleton of non-muscle cells and their expression has distinct consequences on cell morphology. To understand the molecular basis of differences in the function and localization of Tmod isoforms in a cell, we compared the actin filament-binding abilities of Tmod1, Tmod2 and Tmod3 in the presence of Tpm3.1, a non-muscle TM isoform. Tmod3 displayed preferential binding to actin filaments when competing with other isoforms. Mutating the second or both TM-binding sites of Tmod3 destroyed its preferential binding. Our findings clarify how Tmod1, Tmod2 and Tmod3 compete for binding actin filaments. Different binding mechanisms and strengths of Tmod isoforms for Tpm3.1 contribute to their divergent functional capabilities.

1. Introduction

Actin filaments are essential components of the cytoskeleton in cells; they facilitate various processes, such as cell migration, determination of cell shape and muscle contraction (for reviews, see Refs. [19,42]). Actin filaments (F-actin) are formed by polymerization of globular actin (G-actin). Actin filaments have two distinct ends: a fast-growing (barbed) end and a slow-growing (pointed) end. Numerous actin-binding proteins regulate the organization of actin filaments (for review, see Refs. [6,22]). Tropomodulin (Tmod), a 40-kDa tropomyosin (TM)-binding protein, caps the pointed ends of actin filaments [47,48]. Tmods regulate actin dynamics in muscle and non-muscle cells by preventing the polymerization and depolymerization of actin filaments from the pointed ends (for reviews, see Refs. [2,7,50]).

Tmod has two structurally different domains: a disordered N-terminal domain and a compact C-terminal leucine-rich repeat (LRR) domain that consists of a repeated pattern of α-helices and β-strands [12,26,31,33]. The N-terminal domain of Tmod has two TM-binding sites and one actin-binding site [11,16,27,30]. The N-terminal domain of one Tmod molecule binds two molecules of TM at the pointed end [27,29]. TM is a coiled-coil protein that binds along the actin filaments in a head-to-tail fashion (for review, see Ref. [20]). The two TM-binding sites of Tmods have different affinities for TM isoforms [46].

The C-terminal domain of Tmod contains a second actin-binding site [11,17,28,43]. In addition to actin capping, Tmods can sequester actin monomers and nucleate actin polymerization [10,52].

Three Tmod isoforms, Tmod1, Tmod2 and Tmod3, are components of the cytoskeleton of brain cells [4,5]. Tmod1 and Tmod2 have been shown to differently modulate formation of appendages in model cell lines used to study neuritogenesis [9,18,38]. Tmod2 expression level changes in the brain of rats during epilepsy or cerebral ischemia [1,24]. Deletion of Tmod2 results in behavioral changes such as impaired memory and learning, hyperactivity and reduced sensorimotor gating in mice [4].

Tmod isoforms share some cellular compartments. Their structural similarity allows one isoform to compensate for the absence of another. For example, Tmod1 was found to be upregulated after

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Tmod2 knockout in mice [4]. Tmod1 expression increased to compensate for Tmod2 knockdown in neuroblastoma N2a cells [9]. When Tmod1 was knocked out, Tmod3 has been shown to replace it in skeletal muscle cells [15]. Tmod3 is normally not found in red blood cells, but its expression was detected in mouse erythrocytes after Tmod1 knockout [40]. Although a substitution mechanism exists for Tmods, replacement of one isoform with another does not restore normal cell physiology and function [15,40]. Molecular differences between isoforms seem to attribute distinctive functions to Tmods, which affect cellular development and morphology. However, the molecular basis behind these differences in their functions is not very clear.

The purpose of this work was to compare the TM-dependent actin-binding abilities of Tmod1, Tmod2, Tmod3 and study their competition for binding at the pointed ends of actin filaments in the presence of Tpm3.1 (previously TM5NM1, see Ref. [13] for the new TM nomenclature), a non-muscle TM isoform that is involved in neuritogenesis [8] and cell elasticity [25]. Based on data obtained from pyrene-actin polymerization assay, co-sedimentation, cross-linking and non-denaturing PAGE experiments, we propose that cooperative binding of Tmod3 to Tpm3.1 provides an advantage for Tmod3 to bind preferentially to the Tpm3.1-coated actin filaments when in competition with other Tmod isoforms.

2. Experimental procedures

2.1. Protein sequences

Sequences of Tmod1, Tmod2 and Tmod3 were downloaded from UniProt, Ensembl or NCBI for comparison of their amino acid sequences. The accession numbers for the analyzed Tmod1, Tmod2, Tmod3 isoforms are NP_990358.1, NP_001033799.1 and NP_058659.1, respectively.

2.2. Plasmid construction

The plasmids for Tmod1, pET(His)Tmod1 and pET(His)Tmod1$_{1\text{-}344}$, were generated previously [26]. The plasmids for Tmod2, pET(His)Tmod2, and Tmod3, pET(His)Tmod3, were used as templates to obtain pET(His)Tmod2$_{1\text{-}346}$ and pET(His)Tmod3$_{1\text{-}347}$, respectively. pET(His)Tmod3$_{1\text{-}347}$ was then used as a template to construct pET(His)Tmod3$_{1\text{-}347}$[L29E], pET(His)Tmod3$_{1\text{-}347}$[L134D] and pET(His)Tmod3$_{1\text{-}347}$[L29E/L134D]. For site-directed mutagenesis, the plasmids encoding Tmods were amplified by PCR using a set of two complementary oligonucleotides and *Pfu Turbo* DNA polymerase (Agilent Technologies, USA). The original template plasmid was digested with *DpnI* (New England Biolabs), and the mutated plasmid was transformed into *Escherichia coli* (max efficiency DH5α). All designed oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Introduced mutations were confirmed by DNA sequencing at GENEWIZ, Inc. (South Plainfield, NJ).

Tmod2$_{1\text{-}346}$ was generated using an oligonucleotide with the sequence: 5′-CTG GTT CGA AAG AAG AGA GTT TAA GGA GAC AGG AGG-3′. Tmod3$_{1\text{-}347}$ was generated using an oligonucleotide with the sequence: 5′-C AAG AGG CGA ATT TAA GGA GAC CAC CAG TAG CTC G-3′. The L29E mutation was introduced to Tmod3$_{1\text{-}347}$ using an oligonucleotide with the sequence: 5′-CTG TCC GAA TCA GAG GAG AAA CAG CTG GAG ACT GTC C-3′. The L134D mutation was introduced to Tmod3$_{1\text{-}347}$ using an oligonucleotide with the sequence: 5′-G CTG TGC GAC GAT GCA GCT ATT CTT GGG ATG C-3′. The construct for Tmod3$_{1\text{-}347}$[L29E] and the set of oligonucleotides for changing Leu-134 to Asp were used to generate Tmod3$_{1\text{-}347}$[L29E/L134D]. Mutated triplets in the sequences above are underlined.

2.3. Protein expression and purification

WT-Tmod1 and Tmod1$_{1\text{-}344}$ were expressed and purified as described in Ref. [28]. WT-Tmod2 and Tmod2$_{1\text{-}346}$ constructs were overexpressed in *Escherichia coli* BL21 (DE3) using auto-induction medium according to the method described in Ref. [44] and purified using Ni-NTA agarose and ion-exchange chromatography according to the method described in Ref. [38]. Tmod3 constructs (WT, truncated and mutants) were overexpressed in BL21 Rosetta™ 2(DE3)plysS Singles™ cells (Novagen) using LB media with 1% glucose, 0.1 mg/mL carbenicillin and 0.034 mg/mL chloramphenicol that was supplemented with 0.1 mM IPTG for induction at OD_{600} = 0.6. The cells were grown for 5 h after induction and pelleted at 8,000 RPM (Beckman-Coulter JA-10 Rotor), 4 °C, 8 min. The pellets were resuspended in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM Pefabloc and 1 mM TLCK, sonicated and spun down at 16,000 RPM (Beckman-Coulter JA-17 Rotor), 4 °C, 30 min. The pellet that contained Tmod3 in inclusion bodies was resuspended in 50 mM sodium phosphate pH 7.0, 150 mM NaCl and 10 mM Imidazole with 6 M Urea and solubilized overnight at 4 °C on ice. The resuspension was centrifuged at 70,000 RPM (Beckman-Coulter MLA-80 Rotor), 4 °C, 40 min and Tmod3 in the supernatant was purified using Ni-NTA agarose chromatography as in Ref. [26] with denaturing conditions in the presence of 6 M urea. Purified fractions were combined for dialysis against 6 M Urea in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and 1 mM DTT. Urea was removed and the protein refolded by buffer change from 6 M to 0 M Urea in 1 M steps using dialysis. Aggregates were removed by centrifugation at 70,000 RPM (Beckman-Coulter MLA-80 Rotor), 4 °C, 1 h before experiments.

Actin acetone powder was purified as described in Ref. [41]. Chicken skeletal G-actin was purified from acetone powder as described in Ref. [28]. Purified actin was used fresh in fluorescence experiments. Pyrene-iodoacetamide labeled G-actin was prepared [32], labeling ratios were calculated according to [3] and the labeled protein was stored in liquid nitrogen. Pyrene-actin was defrosted at 37 °C and aggregates were removed by centrifugation at 100,000 RPM (Beckman-Coulter TLA-120.1 Rotor), 4 °C, 1 h before experiments.

The construct for rat γ-Tpm3.1 was a generous gift from Dr. Sarah Hitchcock-DeGregori (Rutgers University, Piscataway, NJ), and was expressed and purified as described previously [21,23,37] with modifications. Tpm3.1 construct was overexpressed in *E. coli* BL21 (DE3) using LB media with 0.1 mg/mL carbenicillin that was supplemented with 0.1 mM IPTG for induction at OD_{600} = 0.6. Cells were grown for 5 h after induction and pelleted at 8,000 RPM (Beckman-Coulter JA-10 Rotor), 4 °C, 8 min. The pellet was resuspended in 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 20% sucrose, and 5 mg/mL lysozyme, and frozen at −80 °C for 10 min. After thawing at room temperature, 1 M NaCl was added to the resuspension drop wise, with stirring, and then sonicated for 4 min. The suspension was spun down at 50,000 RPM (Beckman-Coulter MLA-80 Rotor) at 4 °C, 1 h and the supernatant was immersed in boiling water for 5 min. After cooling for 45 min at room temperature, the boiled supernatant was spun at 17,000 RPM (Beckman-Coulter JA-17 Rotor), 4 °C for 30 min. The supernatant was further fractionated by precipitation with $(NH_4)_2SO_4$. At 30% $(NH_4)_2SO_4$ saturation, the mixture was incubated for 20 min at 4 °C and the precipitate was removed by centrifugation at 17,000 RPM (Beckman-Coulter JA-17 Rotor), 4 °C, 30 min. The $(NH_4)_2SO_4$ concentration of the