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Tropomodulin binds to filensin intermediate filaments

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Abstract Tropomodulin (Tmod) is an actin filament pointed end capping protein found in the membrane skeleton of lens fiber cells. We demonstrate that Tmod4 is able to bind the lens-specific intermediate filament protein, filensin, in either co-sedimentation or solid phase binding assays in a saturable fashion, but with low affinity and stoichiometry. Furthermore, Tmod4 does not bind the 53 kDa rod domain of filensin, nor to CP49, the obligate assembly partner of filensin. Finally, the binding of filensin to Tmod4 does not inhibit the actin capping activity of Tmod4 in vitro, suggesting that the two functions are not mutually exclusive.

Key words: Actin; Intermediate filament; Lens; Cytoskeleton; Tropomodulin

1. Introduction

Intermediate filaments have long been viewed as integrators of cellular mechanical strength, and thus important for the integrity of various tissues [1,2]. This structural role is regulated by the activities of intermediate filament-associated proteins (IFAPs), allowing connections to both microtubules and the actin cytoskeleton [2]. In addition, some 'classical' actin binding proteins such as fimbrin have been shown to bind to intermediate filaments or their precursors during cell spreading and movement [3].

The 'beaded' intermediate filaments of the lens are unique in that they are principally comprised of two intermediate filament proteins, filensin and phakinin (CP49), which are not expressed in any other tissue (reviewed in [4]). The restricted expression of these proteins may reflect the unique physical and mechanical requirements of the vertebrate lens, which must be both optically clear and pliable to allow for focusing. These properties arise in part from the ordered and regular organization of the membranes of lens fiber cells, which comprise the bulk of the lens. In the youngest fiber cells of both adult and juvenile lenses, filensin and CP49 appear in a predominantly membrane-associated distribution. In CP49 knockout mice, filensin is completely degraded in the absence of its partner, and the lenses of these mice exhibit increased light scattering properties and dramatically reduced optical quality compared to wild-type littermate controls [5]. Furthermore, the lack of the intermediate filament system results in aberrant fiber cell shapes and plasma membrane organization. These data demonstrate the importance of the filensin intermediate filament system in the functional organization of lens fiber cell membranes.

The actin cytoskeleton is also likely to be important in determining fiber cell shape [6,7], but in contrast to the unique lenticular intermediate filaments, the actin-based membrane skeleton of the lens contains many of the same regulatory proteins found in a variety of cell types. In fact, the biochemical composition of the lens membrane skeleton is remarkably similar to that of the erythrocyte [8], which contains a network of short, tropomyosin-coated actin filaments cross-linked by spectrin molecules into a uniform, regular hexagonal lattice [9,10]. In erythrocytes, the short actin filaments are capped by tropomodulin (Tmod) and directly anchored to spectrin in the membrane skeleton [11]. Unlike the erythrocyte, the lens membrane skeleton is not uniform, and is organized into distinct actin-containing, macromolecular complexes enriched in either spectrin or Tmod, but not both [12]. Tmod is an actin binding protein that specifically regulates the dynamics at the pointed or slow-growing ends of actin filaments. It seems likely that the difference between the organization of Tmod–actin complexes in the lens versus erythrocytes may be due to additional alternate Tmod binding partners in the lens membrane skeleton. Therefore, we sought to identify other potential binding partners for Tmod in the lens membrane skeleton.

2. Materials and methods

2.1. Purification of proteins and in vitro assembly of intermediate filaments

Filensin and CP53 were purified from bovine lenses as described [13]. Recombinant bovine CP49 was expressed from a pT7 expression vector and purified from isolated inclusion bodies [14], using essentially the same chromatography protocol as for filensin and CP53. Purification of recombinant chicken Tmod4 (Sk-Tmod) was performed as described [15]. Polyclonal antibodies to Tmod4 were generated in rabbits and immunoaffinity purified [15].

Assembly of filaments from purified filensin/CP49 or CP53/CP49, or oligomers of filensin alone, was performed as previously described [16] with some modifications [13]. Proteins at 200–500 μg/ml in 8 M urea were dialyzed sequentially into 4, 2, 1, and 0 M urea solutions containing 10 mM Tris pH 8.0, 25 mM β-mercaptoethanol, and 5 mM ethylenediamine tetraacetic acid (EDTA). The final dialysis step was into filament buffer (10 mM Tris pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 25 mM β-mercaptoethanol). The molar ratio of CP49 to either filensin or CP53 was 3:1. Filament assembly for each type (filensin/CP49 or 53kD/CP49) was confirmed by negative staining electron microscopy (data not shown).

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Abbreviations: BSA, bovine serum albumin

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2.2. Sedimentation assays

Purified Tmod4 (in filament buffer) was centrifuged at 300 000 × *g* for 30 min to remove protein aggregates prior to use in sedimentation assays. Assembled filaments (10 μg total protein) were incubated with purified Tmod4 in 100 μl total volume for 20 min at room temperature and then centrifuged at 100 000 × *g* for 30 min through a 30% w/v sucrose cushion. Supernatants were removed, and pellets were resuspended and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 7.5–15% acylamide linear gradient gels. Amounts of Tmod4 in each sample were determined by quantitative Western blot using anti-Tmod4 polyclonal antibodies followed by [^{125}I]protein A [8]. Bands were excised from the nitrocellulose, and counted with a gamma counter. Background bands of equal size were used to subtract non-specific binding of probes. To determine saturation stoichiometry, absolute amounts of Tmod4 and filensin in the pellet were determined by comparing band intensities in Coomassie blue stained gels to bands from known amounts of Tmod4 and filensin on the same gels [8].

2.3. Solid phase binding assays

Solid phase 'dot blot' binding assays were performed using filensin/CP49 filaments or CP53/CP49 filaments. Various amounts of filaments were mixed in filament buffer with 50 μg/ml bovine serum albumin (BSA), and loaded onto nitrocellulose membranes using a DotBlot apparatus (Bio-Rad Industries) [17]. 100 μl of filament solution was loaded per dot, yielding 5 μg BSA and 1–5 μg filensin per dot. Membranes were rinsed in phosphate buffered saline (PBS), and blocked as described [17] prior to probing for 1 h at 4°C with various concentrations of Tmod4 in overlay buffer (10 mM Tris, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2% fish gelatin). Unbound Tmod4 was removed by washing in binding buffer [17], followed by incubation with anti-Tmod4 antibodies followed by [^{125}I]protein A, both in binding buffer. Individual membrane dots were excised and counted with a gamma counter (Beckman). After subtracting background (from control BSA dots incubated with antibody but no Tmod4), counts per minute (cpm) were converted to amounts of Tmod4 using dots loaded with known amounts of Tmod4 and quantitated similarly. For far Western experiments, samples of chicken lens membranes or purified filensin were separated by SDS–PAGE on 7.5–15% acrylamide linear gradient gels and transferred to nitrocellulose. The blots were treated and probed with purified Tmod4 followed by anti-Tmod4 antibodies as described above for the dot blot assays.

2.4. Actin polymerization assays

Measurements of actin polymerization from actin filament pointed ends were carried out using 10% pyrenyl-labeled muscle actin (3 μM actin total) and short gelsolin-capped actin filaments as nuclei (10 nM nuclei; gelsolin:actin ratio of 1:10) [18,19]. Tmod4 samples were preincubated with either filensin oligomers or filament buffer alone for 1 h at room temperature prior to addition to actin and initiation of polymerization assays by addition of salts [19]. Filensin oligomers were used to eliminate contributions of light scattering observed with filensin/CP49 filaments. No significant contribution of light scattering due to the addition of filensin oligomers was observed at the concentrations used here.

3. Results

3.1. Tmod binds specifically to filensin in far Western analyses

To identify potential binding partners for Tmod in the vertebrate lens membrane, chicken lens fiber cell plasma membrane cytoskeleton complexes (PMCC) were isolated, and probed by far Western analyses using purified recombinant Tmod4 followed by anti-Tmod4 antibodies (Fig. 1). The Tmod4 isoform was chosen as a probe, since this is the isoform found in the chicken lens [6]. In chicken lens membranes, a 97 kDa band was detected in addition to the endogenous Tmod4 band at approximately 40 kDa (Fig. 1). This 97 kDa band corresponds to the apparent molecular weight of chicken filensin (CP97, [20]). Purified Tmod4 protein also bound to purified bovine filensin (CP115) in this assay

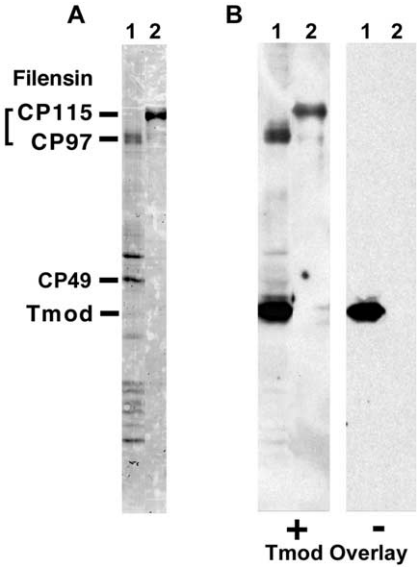

Fig. 1. Tmod4 binds specifically to filensin in the chicken lens PMCC and to purified bovine filensin in far Western blots. Lanes 1, chicken PMCC; lanes 2, purified bovine filensin (CP115). A: Ponceau S stained blot shows total protein electrophoretically transferred to blots. The bands corresponding to purified bovine filensin (CP115), endogenous chicken filensin (CP97), CP49/phakinin (CP49), and Tmod4 (Tmod) are indicated to the left, and were identified in parallel immunoblots (data not shown). B: Far Western blots incubated in the presence (+) or absence (−) of 1 μg/ml recombinant Tmod4. Both blots were probed with an antibody recognizing Tmod4, to reveal both endogenous Tmod4 in the PMCC (+ and −) and recombinant Tmod4 bound to blotted proteins (+).

(Fig. 1). Conversely, other polypeptides observed in the Ponceau stain, including vimentin and CP49, did not show appreciable Tmod4 binding.

3.2. Tmod4 co-sediments with filensin/CP49 filaments assembled in vitro

To determine if Tmod4 could bind to filensin in the context of an assembled 10 nm filament, sedimentation experiments with filaments assembled from purified proteins were performed. 10 nm filaments were assembled as previously described [16] from purified bovine filensin and recombinant bovine CP49 (Fig. 2A). Unlike the endogenous chicken proteins, recombinant bovine CP49 and recombinant chicken Tmod4 were not well resolved by SDS–PAGE (Fig. 2A, lanes 2 and 3). Therefore, quantitative Western blots were performed to assay for Tmod4 co-sedimentation with filensin CP49 filaments (Fig. 2B). A significant amount of Tmod4 co-sedimented with the filensin/CP49 filaments, but not in the absence of filaments (Fig. 2B, lanes 5 and 6). The binding of Tmod4 to filensin/CP49 filaments was saturable, as demonstrated by quantitation of amounts of Tmod4 sedimented as a function of initial Tmod4 concentration (Fig. 2C). These data indicate that the binding interaction is likely to be specific and not due to non-specific association of Tmod4 with the filensin/CP49 filaments. After subtraction of the background amount of Tmod4 sedimented in the absence of filensin/CP49 filaments, the Tmod4 concentration at which half maximal binding occurred was determined to be ~0.4 μM. Comparison of the absolute amount of Tmod4 to filensin by Coomassie blue stained gels of larger samples yielded an approximate satura-

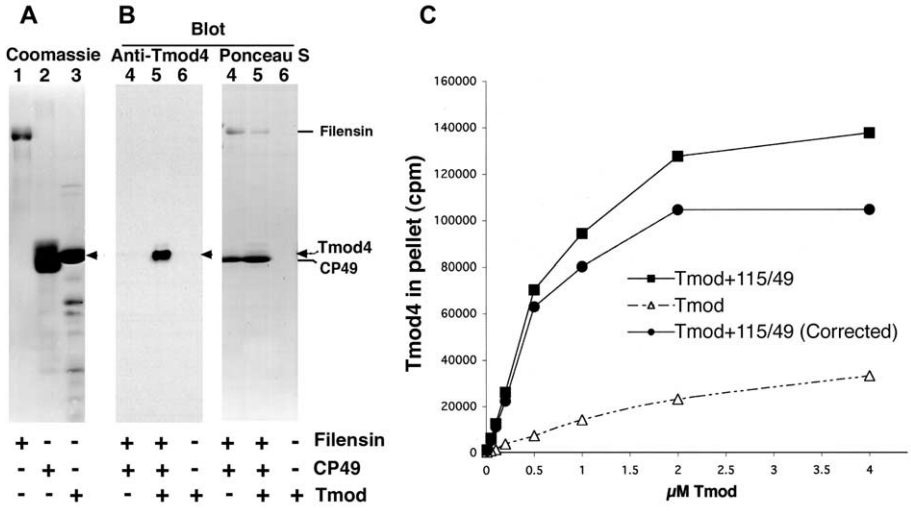

Fig. 2. Tmod co-sediments with filensin/CP49 filaments in solution. A: Coomassie blue stained gel of proteins used for in vitro assembly of filaments and binding by Tmod4. Lane 1, purified bovine filensin (filensin); lane 2, purified bovine phakinin (CP49); lane 3, recombinant chicken Tmod4 (arrowhead). B: Sedimented pellets analyzed by SDS–PAGE followed by blotting to nitrocellulose and staining with Ponceau S (right) or probing with antibodies to Tmod4 (left). Lanes 4, pellet from intermediate filaments sedimented in the absence of Tmod4. Lanes 5, Pellets from intermediate filaments, incubated and sedimented in the presence of Tmod4. Lanes 6, pellets from Tmod4 incubated in the absence of intermediate filaments, and sedimented as above. C: Quantitation of the amount of Tmod4 co-sedimenting with filensin/CP49 filaments, as a function of increasing Tmod concentration. Filled squares, amount of Tmod4 in pellet with filensin/CP49 filaments; open triangles, amount of Tmod4 in pellet in the absence of filensin/CP49 filaments; closed circles, Tmod4 in pellet with filensin/CP49 after subtraction of cpm in Tmod4 alone curve (thus corrected for background of non-specific Tmod4 sedimentation).

tion stoichiometry of 1 mole Tmod4 to 30 moles filensin (data not shown).

Binding of Tmod4 to filensin/CP49 filaments assembled in vitro was also observed using a solid phase 'dot blot' binding assay. In this format, the filaments were assembled as above, and were then spotted onto nitrocellulose membranes (in the presence of 5 μg of BSA to block non-specific interactions; [17]). These substrates were then probed with soluble Tmod4 followed by anti-Tmod4 antibodies and [^{125}I]protein A as for the far Western analyses (Fig. 3). After correction for background (binding of reagents to BSA dots alone), the binding of Tmod4 to filensin/CP49 filaments was observed to be saturable. Moreover, a half maximal binding concentration of $\sim 0.3 \pm 0.15$ μM Tmod4 was observed, in good agreement with the value obtained from the sedimentation assays. Quantitation of the moles of Tmod4 bound per mole of filensin again yielded a low apparent stoichiometry in the range 1:12 to 1:20 (molar ratio of Tmod4 to filensin).

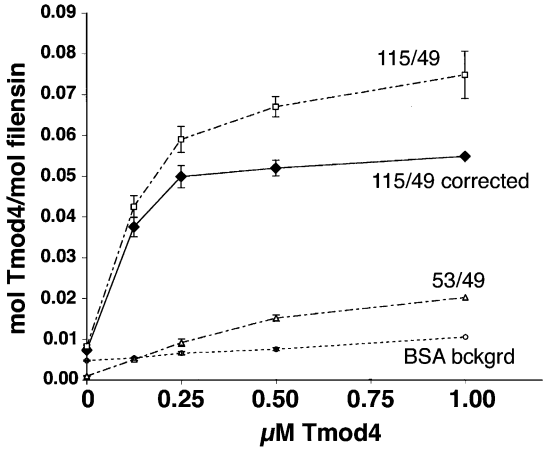

Fig. 3. Tmod4 does not bind to the 53 kDa rod portion of filensin or CP49. Filensin/CP49 or CP53/CP49 filaments were assembled and dotted onto nitrocellulose membranes. Dots were loaded with either filaments assembled with filensin and CP49 (open squares, '115/49'), or with 53 kDa filensin fragment and CP49 (open circles; '53/49'), or without filaments (open triangles, 'BSA bckgrd'). Replicates of dots were probed with various Tmod4 concentrations, and the amounts of Tmod4 bound were measured as described in Section 2. The amount of Tmod4 bound to filensin/CP49 filaments after subtraction of background is indicated by filled diamonds ('115/49 corrected'). Data shown are from triplicate samples, and the experiment is representative of three or more independent experiments.

Previously it has been shown that filensin is proteolytically processed into a polymerization competent 53 kDa rod portion during lens fiber cell aging [21]. This 53 kDa rod (CP53) lacks the C-terminal globular tail domain and a portion of the N-terminal head domain, yet is still competent to form filaments when co-assembled with CP49. To determine if Tmod4 could bind the 53 kDa filensin rod in the context of assembled filaments, the solid phase binding assay was performed using filaments assembled from purified CP53 and CP49. When the Tmod4 bound was normalized to moles of CP53, there was little to no significant Tmod4 binding observed (Fig. 3), suggesting that neither the rod portion of filensin nor CP49 are sufficient to support Tmod4 binding. In agreement with this, we also did not detect binding of Tmod4 to CP53 or CP49 in the far Western analyses (Fig. 1 and data not shown).

3.3. *Filensin binding does not interfere with the ability of Tmod4 to cap pointed ends of actin filaments*

Given that filensin represents an alternate binding partner for Tmod4, an actin capping protein, we wondered whether this binding would preclude the ability of Tmod4 to cap actin filament pointed ends. To investigate this, pyrene-actin polymerization assays were performed [19] to test Tmod4 capping

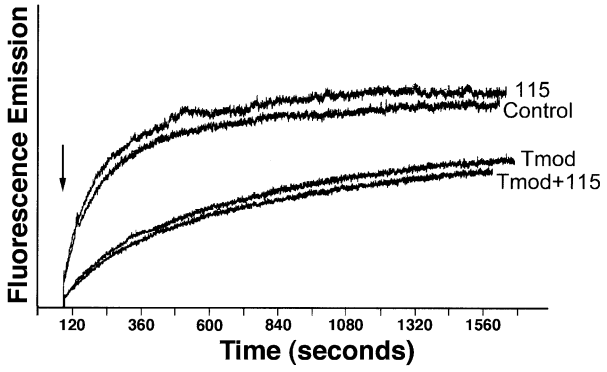

Fig. 4. Filensin binding does not inhibit actin pointed end capping by Tmod4. The effects of Tmod4 on polymerization of actin filaments from pointed ends in the presence or absence of filensin were monitored by pyrene-actin fluorescence (see Section 2). The polymerization reaction was initiated by the addition of polymerization salts at the time indicated by the arrow. 'Control' indicates 3 μM actin alone (no Tmod4 or filensin), 'Tmod' indicates actin polymerized in the presence of 0.4 μM Tmod4, 'Tmod+115' indicates actin polymerized in the presence of 0.4 μM Tmod4 plus 4 μM filensin, and '115' indicates actin polymerized in the presence of 4 μM filensin without Tmod4.

ability, in the absence or presence of stoichiometric amounts of filensin oligomers. When gelsolin-capped short actin filaments are used as nuclei, actin polymerizes from pointed ends only (as monitored by increased pyrene fluorescence, Fig. 4). As expected [15], 0.4 μM Tmod4 reduced the rate of actin polymerization from pointed ends (Fig. 4). However, the addition of a 10-fold molar excess of filensin (4 μM) to the assay had no significant effect on the ability of Tmod4 to slow actin filament elongation from pointed ends (Fig. 4). In fact, no significant differences in Tmod4 activity were observed with filensin concentrations as high as 8 μM (20-fold molar excess, data not shown). The presence of filensin in the absence of Tmod4 had no significant effect on the initial polymerization rates of actin at filament pointed ends. Higher concentrations of filensin could not be used in these assays due to contributions of light scattering, which interfered with pyrene-actin fluorescence. Thus, filensin does not appear to have a significant effect on the pointed capping activity of Tmod4.

4. Discussion

Here we have demonstrated that Tmod4 binds to filensin either as an individual protein (in far Western analyses) or in the context of 10 nm filaments assembled from filensin and CP49 (co-sedimentation analysis and solid phase binding assays). In either solid phase binding assays or in solution sedimentation assays, we observed half maximal binding at a Tmod4 concentration of 0.2–0.5 μM. We have also shown that the binding site for Tmod4 is not contained in the rod domain of filensin, showing that either the tail domain or short head domain of filensin (or both) is required for Tmod4 binding. Tmod4 does not bind to CP49 or vimentin, further suggesting specificity of Tmod4 for filensin. Finally, we have shown that the filensin binding does not significantly inhibit the actin capping activity of Tmod4 in vitro, suggesting that the binding sites for actin and filensin are independent from each other.

The low stoichiometry observed in both far Western and filament binding assays suggests that either the context of the binding site or the presentation of the binding site influences interactions with Tmod4. The possibility of a significant proportion of misfolded or denatured filensin in the assays can be excluded as the purified filensin is competent to assemble in vitro (see Section 2, and [22]). While it is also formally possible that Tmod4 could bind to a contaminating protein present in the filensin fraction rather than filensin itself, no such proteins were detected in the far Western experiments (Fig. 1). We favor the idea that low stoichiometry of Tmod4 binding to filensin/CP49 filaments reflects heterogeneity of the assembled intermediate filaments. Indeed, the mass per unit length of intermediate filaments is known to be variable [23], which would potentially mask many Tmod4–filensin binding sites within a given length of filament. This could also lead to multiple classes of binding sites on filensin/CP49 filaments, which would make estimation of a single dissociation constant not meaningful. Further experimentation will be required to elucidate the nature of the interaction between Tmod4 and filensin.

Previous work has shown that the both the filensin/CP49 intermediate filaments, as well as Tmod are both localized to the lateral membranes of lens outer cortical fiber cells [6,8,24]. Unfortunately, owing to the high density of cytoskeletal elements on the fiber cell membrane and the high concentration of crystallins in the lens, it has not been possible to demonstrate specific co-localization of Tmod4 and filensin/CP49 filaments at the light microscopy level. It is interesting to note, however, that lens membranes extracted with EDTA and Triton X-100 display a paucity of actin and tropomyosin, while Tmod4 and filensin are still present (Fig. 1 and unpublished observations). This suggests that Tmod4 may be retained in this fraction by binding to filensin, in the absence of the tight binding of Tmod4 to tropomyosin-actin [25]. Future work using appropriately genetically engineered mice may elucidate the functional significance of the Tmod4–filensin interaction in vivo.

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