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C-Terminal Variations in β-Thymosin Family Members Specify Functional Differences in Actin-Binding Properties

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Abstract Mammalian cells express several isoforms of β-thymosin, a major actin monomer sequestering factor, including thymosins β4, β10, and β15. Differences in actin-binding properties of different β-thymosin family members have not been investigated. We find that thymosin β15 binds actin with a 2.4-fold higher affinity than does thymosin β4. Mutational analysis was performed to determine the amino acid differences in thymosin β15 that specify its increased actin-affinity. Previous work with thymosin β4 identified an α-helical domain, as well as a conserved central motif, as crucial for actin binding. Mutational analysis confirms that these domains are also vital for actin binding in thymosin β15, but that differences in these domains are not responsible for the variation in actin-binding properties between thymosins β4 and β15. Truncation of the unique C-terminal residues in thymosin β15 inhibits actin binding, suggesting that this domain also has an important role in mediating actin-binding affinity. Replacement of the 10 C-terminal amino acids of thymosin β15 with those of thymosin β4 did, however, reduce the actin-binding affinity of the hybrid relative to thymosin β15. Similarly, replacement of the thymosin β4 C-terminal amino acids with those of thymosin β15 led to increased actin binding. We conclude that functional differences between closely related β-thymosin family members are, in part, specified by the C-terminal variability between these isoforms. Such differences may have consequences for situations where β-thymosins are differentially expressed as in embryonic development and in cancer. J. Cell. Biochem. 77:277–287, 2000.

Key words: thymosin β isoforms; mutational analysis; cytoskeleton; prostate cancer; actin

The regulation of actin is crucial for cellular functions ranging from mitosis to motility as seen in embryology, in wound healing, or in gain of metastatic potential [Stossel, 1993; Devineni et al., 1999; Fidler et al., 1978; Zetter, 1990]. It is clear that such functions depend on the tight regulation of actin polymerization. The actin monomer sequestering proteins are critical to this regulation. We previously identified thymosin β15, a novel member of the actin monomer sequestering β-thymosin family as a molecule that is upregulated in aggressive human prostate cancer [Bao et al., 1996]. Thymosin β15 is expressed in highly motile, metastatic prostate cancer cells, as well as in advanced human prostate and breast cancer [Bao et al., 1996; Gold et al., 1997]. Thymosin β15 was not found in normal prostate or breast tissue and was not upregulated in benign prostate hyperplasia [Bao et al., 1996; Gold et al., 1997]. Furthermore, we showed that thymosin β15 expression correlates with motility and metastasis in highly metastatic prostate carcinoma cells. Although the normal and carcinoma cell lines also express related β-thymosins (β4 and β10), their expression did not correlate with metastatic potential or with altered cellular motility. This finding suggests that thymosin β15 differs from other β-thymosins, conceivably allowing it to regulate cellular motility and perhaps metastatic potential through its ability to bind G-actin and retard actin polymerization.

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Actin, the most abundant cytoskeletal protein and a key player in cellular motility, exists in two states in a cell: monomeric actin (G-actin) and filamentous actin (F-actin) [Fechheimer and Zigmond, 1993]. The assembly of F-actin is dependent, in part, on the equilibrium between G- and F-actin. This equilibrium is maintained partially by actin monomer binding proteins that sequester G-actin [Sun et al., 1995; Carlier and Pantaloni, 1997]. The β-thymosin family members are the most abundant of the monomer binding proteins [Cassimeris et al., 1992; Safer and Nachmias, 1994]. Currently, there are at least 16 identified β-thymosins. Thymosin β4, the most thoroughly studied member, is expressed ubiquitously in mammalian cells [Yu et al., 1993; Jean et al., 1994], whereas the expression of thymosin β10 is distributed more selectively with levels modulated in embryological development and disease [Verghese-Nikolakaki et al., 1996; Carpintero et al., 1996; Califano et al., 1998]. The family of β-thymosins share approximately 62–68% amino acid sequence homology. With the high degree of homology among the large number of β-thymosin family members, it is unclear how these molecules differ functionally and why there are such diverse patterns of expression. The structural interactions between thymosin β4 and actin have been studied, but there is limited information regarding functional differences in actin-binding or other properties between the different β-thymosin family members.

Several groups have identified key domains of thymosin β4 involved in actin binding. The N-terminus contains an important α-helical domain, residues 5–16 [Czisch et al., 1993; Feinberg et al., 1996]. When this structure is disrupted by the insertion of a proline at position 11 (K11P) [Van Troys et al., 1996], or when the region is truncated by 6 or12 amino acids [Vancompernolle et al., 1992; Huff et al., 1995], actin-binding is severely diminished. Another essential domain is the central conserved motif 17-LKKTETQ-23, a conserved domain shared by several monomeric actin-binding proteins, including the β-thymosins and actobindin [Vancompernolle et al., 1991]. The actin-binding activity of thymosin β4 is severely compromised by mutation of leucine 17 to alanine (L17A) [Van Troys et al., 1996]. The C-terminus shows the greatest heterogeneity between different members of the β-thymosin family. Truncations of this region decrease the actin-binding affinity of thymosin β4. Initial work by Vancompernolle et al. [1992] showed a decreased actin-binding affinity of 25- to 50-fold with truncations of the C-terminal 13 or 19 amino acids in thymosin β4. Similarly, Huff et al. [1997] reported that the loss of the last two C-terminal amino acids decreased the actin-binding affinity of thymosin β10.

Structural analysis of the β-thymosin-actin complex has been performed using magnetic resonance imaging (MRI) [Czisch et al., 1993; Safer et al., 1997]. Safer and coworkers [1997] showed that thymosin β4 binds actin in an extended conformation making intimate contact along the entire length of the β-thymosin molecule. This close association emphasizes the importance of the entire β-thymosin molecule and suggests that small alterations in a β-thymosin sequence could alter the actin-binding characteristics and consequently influence the cellular phenotype. Consequently, variations anywhere along the length of the molecule could theoretically result in altered actin-binding potential among different β-thymosin isoforms.

Thymosin β15 differs from other β-thymosin isoforms in the conserved central motif and, more substantially, at the C-terminus. To explore the hypothesis that thymosin β15 might have altered actin-binding properties that could influence the phenotype of cancer cells, we compared the actin-binding affinities of thymosin β15 and thymosin β4. We found significant reproducible differences in actin-binding affinities between these two β-thymosin isoforms. Consequently, we conducted mutational analysis of thymosin β15 to determine the specific domains responsible for the observed changes in actin-binding (Fig. 1). We conclude that although multiple

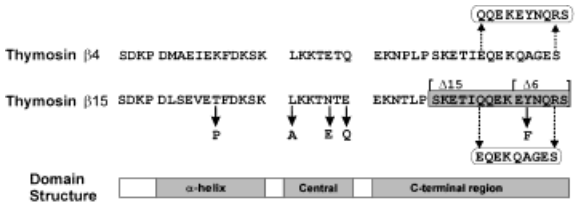

Fig. 1. Sequences of thymosin β15 and β4 with mutations used in this study. The three principal domains of the β-thymosin family members—the α-helix, the conserved central domain, and the C-terminal region—are illustrated and labeled in the diagram. Solid arrows, point mutations in thymosin β15. Thymosin β15 C-terminal truncations of 15 and 6 amino acids are bracketed and labeled Δ15 and Δ6. Dashed arrows, thymosin β15/β4 C-terminal swaps; in boxes, amino acids.

β-thymosin domains participate in actin-binding activity, subtle amino acid variations in the C-terminal domain specify important functional differences between β-thymosin isoforms.

MATERIALS AND METHODS

Cloning of β-Thymosin-GST Fusion Proteins

The genes encoding thymosin β15 and β4 were cloned into a pGEX-2T vector to produce a β-thymosin-GST fusion protein (Pharmacia Biotech, Piscataway, NJ), obtained from Dr. Lere Bao (Children's Hospital, Boston, MA). The β-thymosin mutants were constructed by polymerase chain reaction (PCR)-based site-directed mutagenesis (Stratagene, La Jolla, CA), using DNA primers (Gibco-BRL, Gaithersburg, MD) that contained the appropriate point mutations. For the two C-terminal hybrid mutants, Tβ15-β4C and Tβ4-β15C, silent mutations were first incorporated to introduce restriction sites that facilitated subsequent cleavage and ligation of the C-terminal ends. *Epicurian coli* XL1-Blue supercompetent cells (Stratagene) were transformed with plasmids, ampicillin-resistant clones were confirmed to contain the correct mutations by restriction analysis screening and subsequent DNA sequencing (Molecular Biology Core Facilities, Dana Farber Cancer Institute, Boston, MA).

Expression and Purification of β-Thymosin-GST Fusion Proteins

Cells containing the β-thymosin pGEX-2T plasmids were grown in LB-Medium (Bio 101, Vista, CA) and ampicillin at 37°C; 1-L cultures were inoculated, grown to an optical density (OD) of 0.6; protein expression was then induced with isopropyl-β-D-thiogalactopyranoside. After 6 h, cells were pelleted, resuspended in 50 ml phosphate-buffered saline (PBS), sonicated on ice, and centrifuged at 12,000*g* for 10 min. The supernatant containing the GST-fusion proteins was incubated with a slurry of glutathione-agarose beads (Pharmacia Biotech). After extensive washes with PBS, the proteins were cleaved from the GST tag with 60 U of thrombin (Pharmacia Biotech). The purity of the cleaved β-thymosin proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The cleaved β-thymosin proteins were later dialyzed in 50 mM ammonium acetate using Slide-A-Lyzer cassettes with a 3,500-M_r weight cutoff (Pierce). Protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Aliquots of protein were dried on an ISS110 SpeedVac (Savant, Holbrook, NY) and then stored with desiccant at −20°C.

Nomenclature: Amino Acid Sequence Numbering of Cleaved β-Thymosin Recombinant Proteins

The recombinant β-thymosin proteins were enzymatically cleaved from the GST N-terminal tag, resulting in the addition of a glycine and serine to the N-terminal methionine. To remain consistent with previous work on β-thymosin proteins, the second serine was assigned position 1.

Actin Monomer Sequestering Assay

We used fluorescence enhancement after binding of tetramethylrhodamine isothiocyanate-labeled phalloidin (TRITC-phalloidin) to F-actin [De La Cruz and Pollard 1994; Huang et al., 1992; Allen and Janmey 1994], to assay F-actin concentration in the presence and absence of various β-thymosin isoforms. β-thymosin proteins were resuspended in G Buffer (0.2 mM $CaCl_2$, 2.0 mM Tris, pH 8.0, 0.5 mM ATP, 0.5 mM DTT) to an approximate concentration of 1.0 mg/ml. The protein concentration was confirmed with the BCA Protein Assay kit. To assay actin polymerization, 0–5.0 μM monomeric actin was combined with 5.0 nM gelsolin, and 4.0 μM β-thymosin in 50 μl of F buffer (0.2 mM $CaCl_2$, 2.0 mM Tris, pH 8.0, 0.5 mM ATP, 0.5 mM DTT, 150 mM KCl, 2.0 mM $MgCl_2$). After an overnight incubation at 4°C to allow polymerization, the mixture was diluted 10-fold into G-buffer containing 1.0 μM TRITC-phalloidin (Sigma Chemical Co., St. Louis, MO). After a ten min incubation, the fluorescence intensity was measured using the plate reader accessory of the Perkin-Elmer LS50B fluorescence spectrophotometer (Norwalk, CT). A wave-length of 540 nm was used to excite the TRITC-phalloidin and the light emitted at 575 nm was measured.

The assay provides results equivalent to that using pyrene iodoacetamide-derivatized actin (data not shown), requires less volume per condition and can be used without chemical modification of actin [Allen et al., 1996]. The use of a 96-well plate reader to assay fluorescence also increases assay throughput. While phalloidin can drive the polymerization of β-thymosin-actin

complexes [Reichert et al., 1994], under our conditions, the fluorescence intensity and F-actin concentration was stable for at least 30 min (data not shown).

Cloning the β-Thymosin-EGFP Fusion Protein

To create β-thymosin-EGFP (enhanced green fluorescence protein) fusion proteins, wild-type and mutant β-thymosin sequences were cloned into pEGFP-C1 (Clontech, Palo Alto, CA). DH5α competent cells (Gibco-BRL) were transformed with these plasmids, and positive clones were selected with kanamycin.

Cell Culture and Transfection

The Dunning R-3327 rat prostatic adenocarcinoma cell line AT2.1 (obtained from J. Isaacs, Johns Hopkins University) was used in transfection studies. Cells were grown in RPMI (Gibco-BRL) supplemented with 10% fetal calf serum (FBS) (Gibco-BRL), 1.0% glutamine/penicillin/streptomycin (Gibco-BRL), and 250 nM dexamethasone (Sigma). Cells were plated 1:50 on glass coverslips that had been pretreated with sequential incubations in 1.0 M hydrochloric acid for 4–12 h at 55°C, 1.0 mg/ml poly-L-lysine (Sigma) for 30 min at room temperature, and 10 μg/ml fibronectin (Collaborative Biomedical Products, Bedford, MA) for 30 min at room temperature. At 24 h postseeding, the cells were transfected with 1.0 μg β-thymosin-EGFP wild-type or mutant plasmid DNA, using Lipofectamine Plus (Gibco-BRL).

Rhodamine-Phalloidin Staining

At 24 h post-transfection, AT2.1 cells were fixed with 4.0% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) and permeabilized in 0.2% Triton X-100 (Sigma). The cytoskeleton was stained with a 1:100 dilution of rhodamine-phalloidin (Molecular Probes, Eugene, OR) in 0.2% Triton X-100/0.2% bovine serum albumin (BSA) in PBS. Coverslips were mounted on glass slides with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and stored at −20°C. Rhodamine and EGFP were viewed using a Zeiss Axiophot D-7082 microscope (Carl Zeiss, Thornwood, NY). The cell fields were photographed under consistent exposure conditions. Cells expressing thymosin β4, β15, and mutant β15-EGFP fusion protein with equivalent fluorescence levels were compared, and changes to the stress fibers were evaluated.

RESULTS

Prostate cancer cells that express thymosin β15 differ in their morphology, motility, and metastatic potential compared with cells that do not express thymosin β15 [Bao et al., 1996]. Unlike thymosin β15, thymosin β4 expression does not correlate with metastatic potential in these tumor cells. To investigate our hypothesis that the highly motile and metastatic phenotype might be related to the actin-binding properties of thymosins β15 and β4, we began by determining whether thymosin β15 acted to sequester actin monomers and then compared the actin-binding properties of thymosin β15 to those of thymosin β4.

Thymosin β15 Has Higher Actin-Binding Affinity Than That of Thymosin β4

β-Thymosins sequester actin with micromolar affinity and share characteristic residues and regional conformation vital for actin binding. Purified recombinant thymosins β15 and β4 were assayed in an actin monomer sequestering assay to determine their actin-binding affinities. Both β-thymosins inhibited actin polymerization, but thymosin β15 inhibited polymerization to a greater degree than thymosin β4 (Fig. 2). The calculated kD for thymosin β4 binding to G-actin was 4.8 μM, whereas thymosin β15 had an increased actin-binding affinity with a kD of 2.0 μM. The calculated kD for thymosin β4 in this study was consistent with previously reported values [Yu et al., 1993; Van Troys et al., 1996; Weber et al., 1992].

Actin-Binding Affinities Correlate With Cellular Stress Fiber Density

Cells transiently transfected with thymosin β4 have been observed to reduce the number of visible stress fibers [Yu et al., 1994; Safer and Nachmias, 1994; Weber et al., 1992]. We tested whether the increased actin-binding affinity of thymosin β15 would lead to a decrease in levels of cellular filamentous actin. Dunning rat AT2.1 cells were transiently transfected with vectors encoding β-thymosin-EGFP fusion proteins and were subsequently stained with rhodamine-phalloidin. The cells were evaluated for degree of vector expression by comparing cellular EGFP fluorescence levels. As shown in Figure 3, cells expressing high concentration of thymosin β15 showed reduced staining with rhodamine-phalloidin.

We then compared the ability of thymosin β4 and β15 to reduce intracellular filamentous actin levels. Both thymosins β4 and β15 transfected cells had reduced stress fibers compared with control. However, cells expressing thymosin β15 had consistently fewer visible stress fibers than those expressing equivalent concentrations of thymosin β4 (Fig. 4).

Mutations to the α-Helix and Conserved Central Motif Impair Actin-Binding and Do Not Alter Cellular Filamentous Actin Concentrations

N-terminal α-helical residues 5–16 [Czisch et al., 1993; Feinberg et al., 1996] and the conserved central motif 17-LKKTETQ-23 [Vancompernolle et al., 1992] have previously been identified as important domains for β-thymosin actin binding. Mutations have been made to thymosin β4 α-helix position 11 (K11P), and position 17 (L17A) within the conserved central motif. Both mutations have been shown to inhibit actin binding independently [Van Troys et al., 1996]. Identical mutations in thymosin β15 were made. Thymosin β15-T11P had no inhibitory activity at concentrations where wild-type thymosin β15 inhibited actin polymerization (Fig. 5A). This finding supports the hypothesis that formation of an α-helical structure by the N-terminus of thymosin β15 is necessary to inhibit actin polymerization. Similarly the L17A mutation in the conserved central motif was unable to inhibit actin polymerization, in contrast to wild-type thymosin β15 (Fig. 5B). Cells transfected with these mutant β-thymosins had no visible alterations in stress fiber density (data not shown). The actin-binding and cellular transfection results confirmed that thymosin β15 behaved similarly and shared characteristic essential functional residues in the N-terminal and central domains previously reported for other β-thymosin family members.

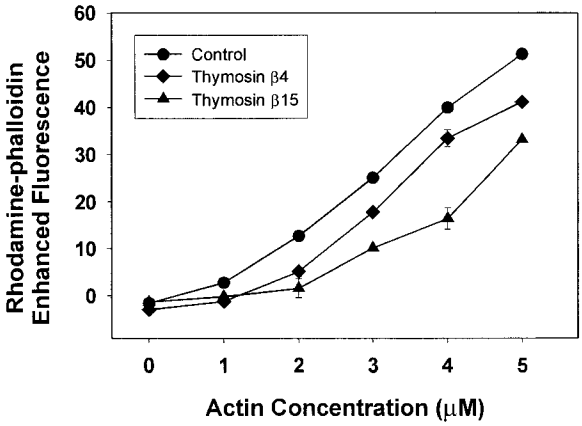

Fig. 2. Comparison of the actin-sequestering activity of thymosins β15 and β4. Effect of β-thymosins on the final levels of F-actin was measured from the enhanced fluorescence of rhodamine-phalloidin upon binding to actin filaments, as described under Materials and Methods. β-Thymosin protein was added to variable concentrations of G-actin in the presence of 5 nM gelsolin. Gelsolin was added to ensure that polymerization occurred at the low-affinity, or pointed, end. The conditions were 4.0 μM thymosin β15 (▲), 4.0 μM thymosin β4 (◆), or β-thymosin-free control (■). Error bars represent standard error of the mean (n = 3).

Mutations to the Conserved Central Motif Do Not Alter the Actin-Binding Affinity of Thymosin β15

The observation that thymosin β15 had higher actin-binding affinity than that of thymosin β4 suggested that, although these two molecules share significant amino acid sequence homology, the few sequence variations that exist must influence the actin-binding activity. Consequently, we identified unique amino acid residues and unique domains in thymosin β15. A series of

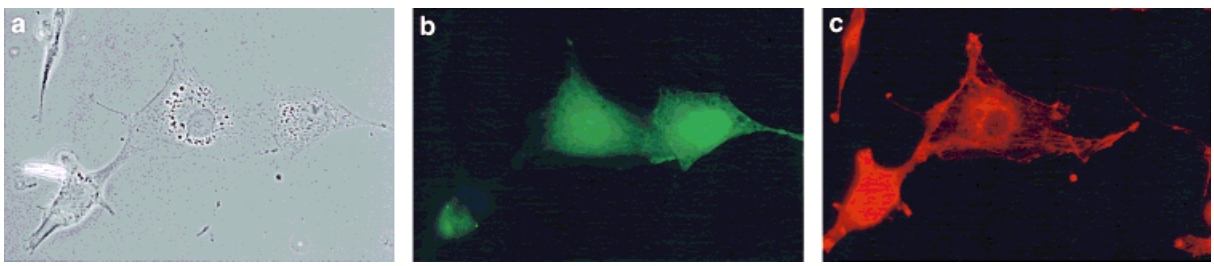

Fig. 3. Effect of thymosin β15 transfection on filamentous actin levels in prostate carcinoma cells. Cells transfected with thymosin β15-EGFP show reduced levels of filamentous actin, as demonstrated by rhodamine-phalloidin staining. **a:** Phase microscopy. **b:** EGFP staining reveals increased levels of EGFP-thymosin β15 in some transiently transfected cells. **c:** Cells with highest thymosin β15 concentrations showing reduced levels of filamentous actin.

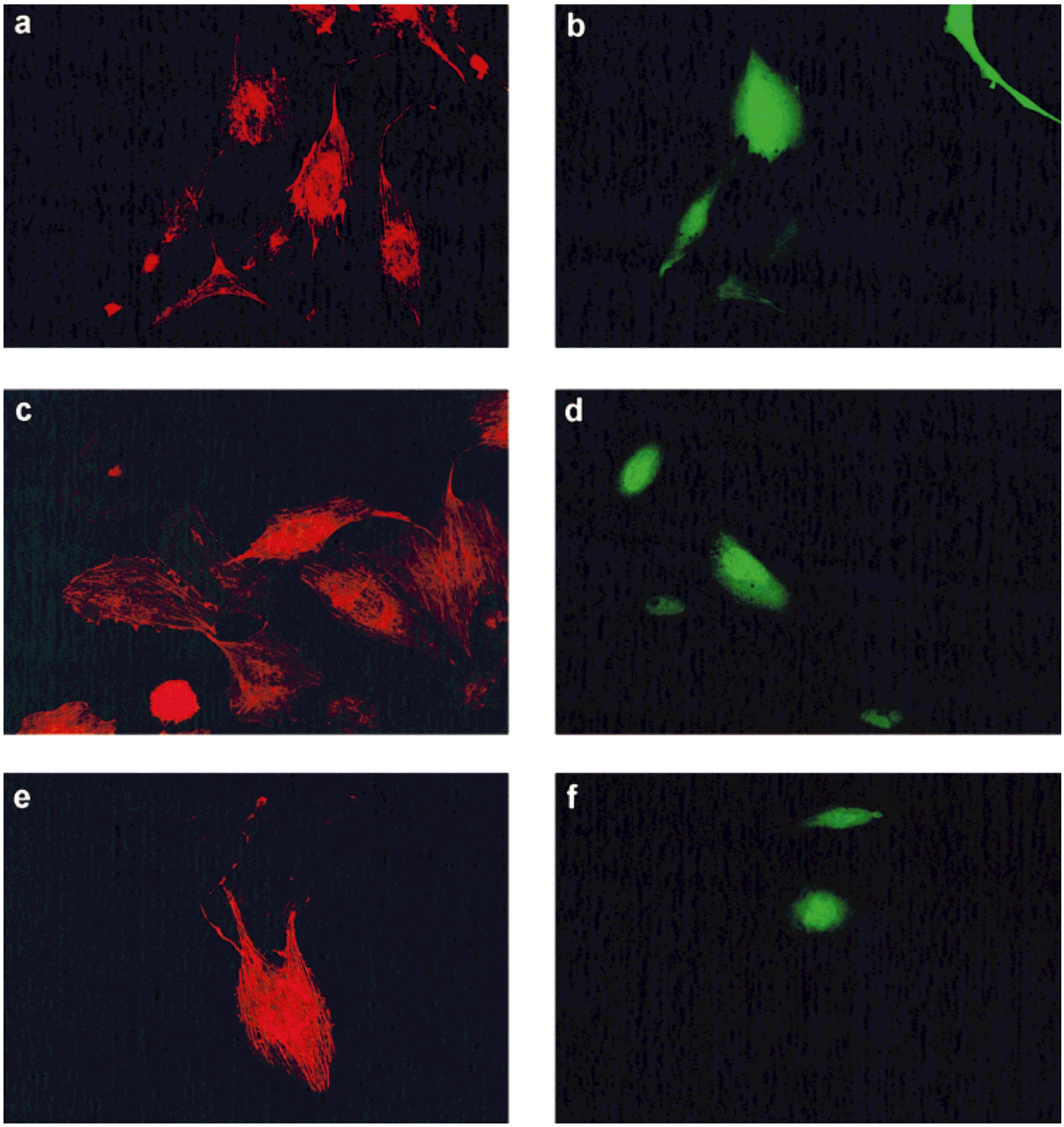

Fig. 4. Effect of transfection with β-thymosin isoforms. Dunning rat AT2.1 cells were transfected with 1.0 μg of EGFP alone (**a,b**), thymosin β4-EGFP (**c,d**), or thymosin β15-EGFP plasmid cDNA (**e,f**). At 24 h post-transfection, cells were fixed, stained with a 10^{-2} dilution of rhodamine-phalloidin, and then viewed. Cells with similar EGFP fluorescence levels were examined (**b,d,f**) and the rhodamine-stained cytoskeletons compared (**a,c,e**). Identical fields: **a,b**, **c,d**, and **e,f**.

point mutations, truncations, and domain swaps were created. Our goal was to identify regional motifs responsible for the enhanced actin-binding activity of thymosin β15.

The importance of the conserved central motif 17-LKKTETQ-23 in β-thymosin-actin interactions has been reported by several groups [Van Troys et al., 1996; Vancompernolle et al., 1992; Huff et al., 1997]. This motif is conserved within the β-thymosin family, except for thymosin β15, which has unique amino acid residues at position 21 and 23 (17-LKKTNTE-23). To determine whether these residues were responsible for the increased actin-binding affinity of thymosin β15, three mutants were designed to recreate the conserved central motif.

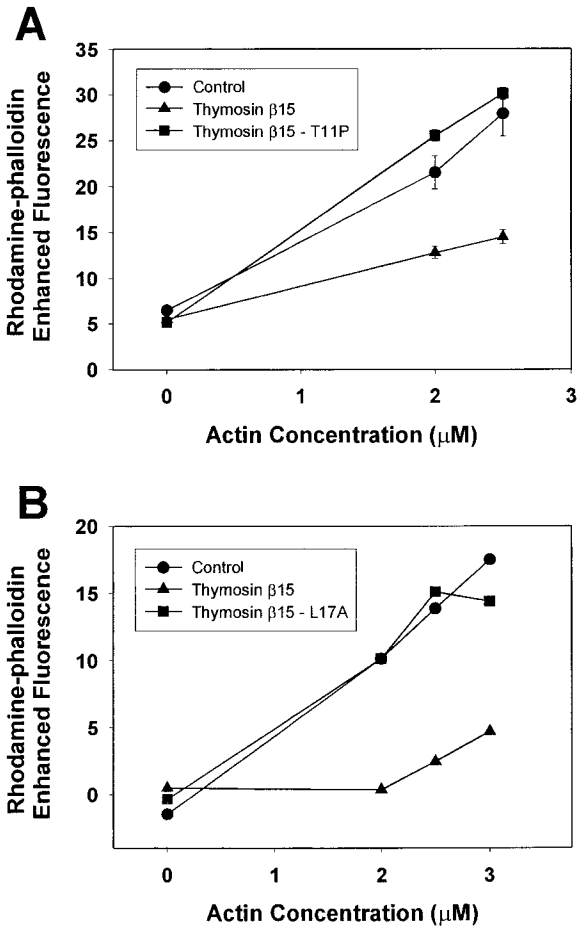

Fig. 5. Residues critical for β-thymosin-actin interaction are retained in thymosin β15. Mutations to the α-helix (K11P) and conserved central motif (L17A) have been shown to inhibit actin-binding activity of thymosin β4. Similar mutations generated in thymosin β15 (**A**) T11P (■) and (**B**) L17A (■) were tested for their actin-binding activity, as described under Materials and Methods. At 4.0-μM concentrations of β-thymosin mutants, actin-binding activity was not detected. Mutants were compared with β-thymosin-free control (●), and thymosin β15 (▲).

Each amino acid was changed both individually from that in thymosin β15 to the conserved motif (N21E and E23Q) and together (N21E/E23Q). The mutants were evaluated in the actin monomer sequestering assay and compared with wild-type thymosin β15. All three mutants showed no significant alteration in actin-binding affinity compared with thymosin β15 wild type (Fig. 6). These results suggest that the nonconserved residues in the conserved central domain of thymosin β15 are not responsible for the observed alterations in actin-binding affinity.

The C-terminal domain of β-thymosins specifies actin-binding affinity. The C-terminus has

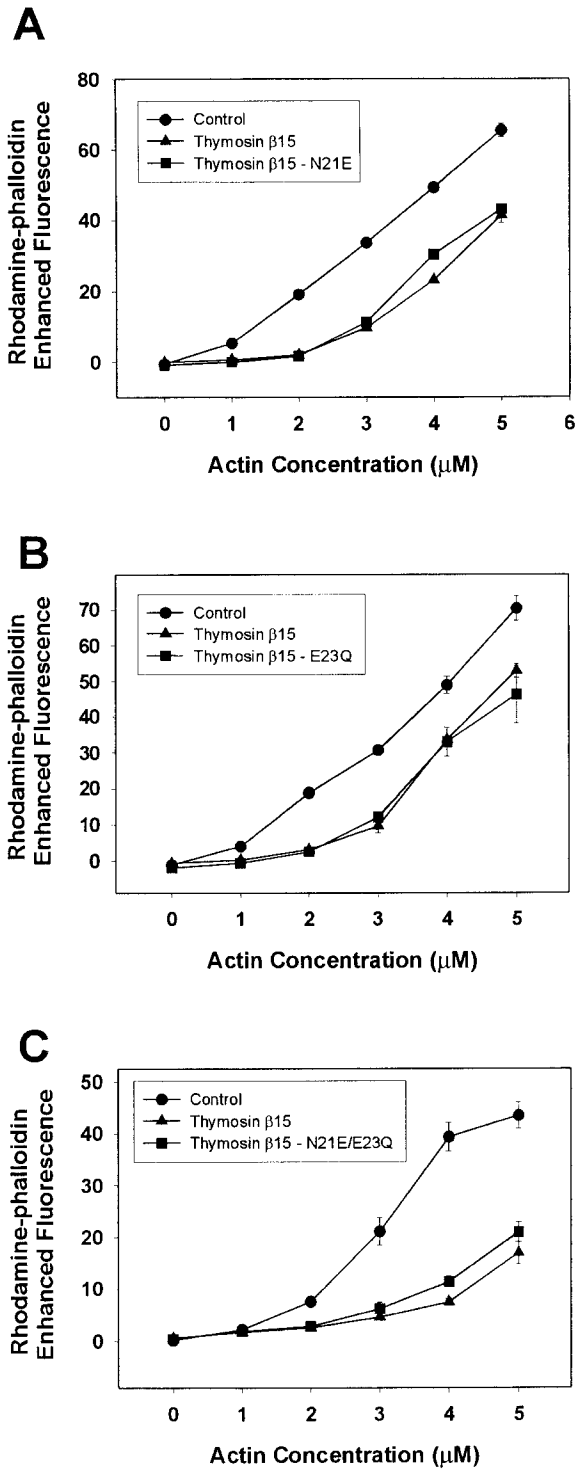

Fig. 6. Variations in the conserved central domain unique to thymosin β15 do not explain the differences in the actin sequestering activity between thymosin β15 and β4. Mutations were made in thymosin β15 to test the individual residues (**A**) N21E (■), (**B**) E23Q (■), as well as to replicate the classic conserved central motif 17-LKKTETQ-23 (**C**) N21E/E23Q (■). Actin-sequestering activity was assayed as described under Materials and Methods. Conditions were 4.0 μM thymosin β15 (▲), 4.0 μM thymosin β15 mutants (■), or β-thymosin-free control (●).

the highest degree of variability within the β-thymosin family. Thymosins β4 and β15 are highly homologous, except for the last 5 and 6 amino acids, respectively. We therefore created two C-terminal truncations and evaluated them in the actin-sequestering assay. Initially, we removed a large portion of the C-terminus of thymosin β15 to evaluate its importance for actin binding. The mutant (thymosin β15-Δ31–44), lacking the last 15 residues, was unable to sequester actin at concentrations where wild-type thymosin β15 was active (Fig. 7A). Next, we selectively truncated the unique six terminal amino acids of thymosin β15 39-EYNQRS-44 (thymosin β15-Δ39–44). This mutant also showed no ability to inhibit actin polymerization under our conditions (data not shown). These results indicate that the intact C-terminus is vital for actin-binding, and that the heterogeneity within the β-thymosin family at the C-terminus may modify actin binding.

Most β-thymosins lack tyrosine residues. We therefore targeted the tyrosine at position 40 in thymosin β15 to determine whether this single amino acid was responsible for any of the enhanced actin-sequestering capacity of thymosin β15. Substitution of phenylalanine for tyrosine caused no alteration in the ability of the molecule to interact with actin (data not shown). Thus, the tyrosine at position 40 is not responsible for the increased actin-sequestering activity of thymosin β15.

As the unique tyrosine was not responsible for actin-binding affinity differences, we further investigated the specific contribution of the C-terminus to actin affinity. A C-terminal hybrid thymosin β15-β4C was created (thymosin β15 1–34/β4 35–43) to address whether exchanging the last ten C-terminal amino acids of thymosin β15 with those of thymosin β4 would alter the actin-binding affinity of the new hybrid molecule. Intriguingly, the hybrid

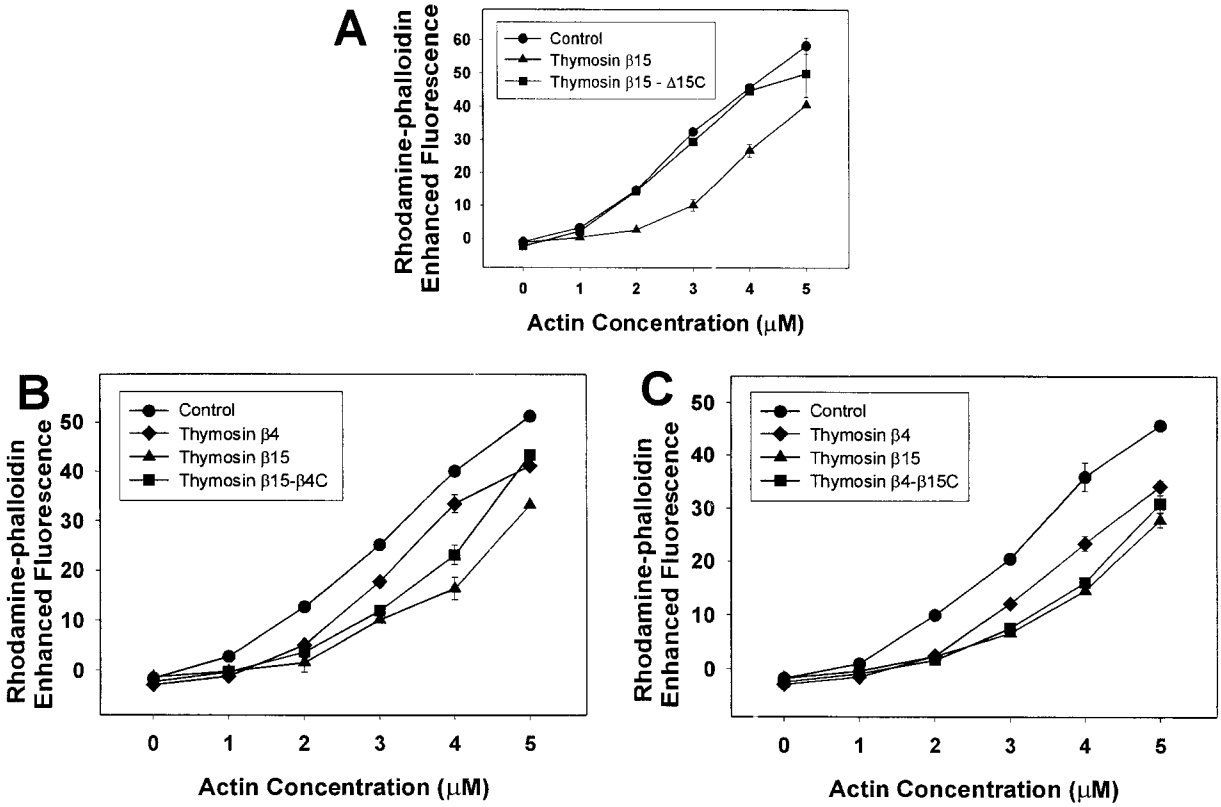

Fig. 7. Effect of C-terminal mutations on actin binding. The importance of the C-terminus for β-thymosin actin-binding was investigated with truncational and hybrid mutants. Truncation of the C-terminal 15 amino acids in thymosin β15 (**A**) thymosin β15-Δ31–44 (■) disrupted actin-binding activity. The C-terminal hybrid mutants (**B**) thymosin β15 1–34/β4 35–43 (■), and (**C**) thymosin β4 1–34/β15 35–44 (■) showed actin-binding affinities intermediate to those of thymosin β15 and β4. Actin-binding activity was assayed as described under Materials and Methods. Conditions were 4.0 μM thymosin β15 (▲), 4.0 μM thymosin β4 (◆), 4.0 μM β-thymosin mutant (■), and thymosin-free control (●).

thymosin β15-β4C showed decreased actin-sequestering activity relative to wild-type thymosin β15 (Fig. 7B). The dissociation constant was 3.2μM, which was intermediate to those of wild-type thymosin β15 and β4.

If the C-terminal domains of thymosin β4 and β15 are partially responsible for the differences in actin-affinity between the two molecules, the complementary experiment in which the C-terminus of thymosin β4 is replaced with that of thymosin β15 (thymosin β4 1–34/β15 35–44) would be expected to show increased actin-binding affinity relative to wild-type thymosin β4. As shown in Figure 7C, the thymosin β4-β15C hybrid had increased actin-binding affinity as compared with wild-type thymosin β4, although less than wild-type thymosin β15. These results demonstrate that C-terminal variations among different members of the β-thymosin family can lead to altered actin-binding and sequestering activities.

DISCUSSION

β-Thymosins are thought to be the predominant actin sequestering proteins in most cells [Cassimeris et al., 1992; Yu et al., 1993]. At least 16 family members exist, and several isoforms can be expressed within the same cell. Recently, we identified a novel β-thymosin isoform, thymosin β15, expressed in metastatic prostate carcinoma cells and demonstrated that reduced thymosin β15 expression caused reduced cell motility in these cells [Bao et al., 1996]. To begin to define how this molecule influences cytoskeletal function, we have characterized its interactions with its only known ligand, actin. Direct comparison of actin-sequestering activity demonstrates that thymosin β15 binds more tightly to actin than the more widely distributed thymosin β4. Mutational analysis leads to the conclusion that C-terminal sequence variations between these two molecules account for a substantial portion of the differences in actin affinity between these two isoforms. These observations support a structural model of β-thymosin-actin interaction and provide insight into the general mechanisms underlying cell motility.

Thymosin β4 is the most extensively investigated β-thymosin. Mutational analysis and NMR studies have clearly identified important contributions of the N-terminal α-helix [Czisch et al., 1993; Van Troys et al., 1996], the conserved central domain 17-LKKTETQ-23 [Vancompernolle et al., 1992; Huff et al., 1997], and a less clearly defined contribution by the C-terminal region [Vancompernolle et al., 1992] to β-thymosin-actin binding. A preliminary model for the actin-thymosin β4 complex proposed by Safer et al. [1997] indicates all three domains of thymosin β4 are in close contact with actin, suggesting that minor changes in any of these domains might alter actin binding. Consequently, we have investigated the role of each of these domains in modulating the actin-sequestering activity of thymosin β15.

Structural Insights Into β-Thymosin-Actin Interaction

Mutagenesis studies of β-thymosin interaction with actin have focused significant attention on two regions of this molecule. NMR studies of the β-thymosin peptide have suggested that it can be induced to form a helix toward its N-terminus [Czisch et al., 1993; Safer et al., 1997]. The importance of this coil to helix transition for normal interaction with the actin monomer was confirmed by several studies, which demonstrated that introducing helix-breaking mutations, such as K11P [Van Troys et al., 1996] into this N-terminal domain disrupts normal actin sequestering activity. Similarly, a conserved region (residues 17–23) was identified as an "actin-binding motif" based on homology to regions in other actin-binding proteins [Vancompernolle et al., 1992]. Point mutations within this region significantly disrupted thymosin β4-actin interactions [Van Troys et al., 1996]. Our current results from mutagenesis of thymosin β15 at positions 11 and 17 validate the importance of both the α-helical and central domains of the molecule in mediating interactions with actin.

Functional Contribution of the C-Terminal Domain

The functional role of the β-thymosin C-terminal domain has not been well characterized. Recent modeling of the thymosin β4-actin interaction using NMR data by Safer and colleagues [1997] suggest that both the N- and C-termini of thymosin β4 make contact with actin, though in very different places. C-terminal truncations in thymosin β4 produce a 25- to 50-fold decrease in actin-binding affinity, indicating that the C-terminal domain makes a functional contribution to actin-binding [Vancompernolle et al., 1992].

Our mutational analysis provides further evidence that the C-terminus of the β-thymosins is

important to β-thymosin-actin interactions. Truncation of just six C-terminal residues caused a complete loss of actin sequestration under our assay conditions. Importantly, our results from C-terminal swapping experiments reveal that alterations at the C-terminus contribute to the functional differences in actin-binding between different β-thymosin isoforms. This finding is important because the thymosin β family is highly conserved and differences between the various isoforms are largely confined to the C-terminal domain whereas differences in the N-terminal or central domains are relatively rare. C-terminal amino acid variations do not, however, account completely for the actin-binding differences since the C-terminal swaps had actin-sequestering activities intermediate to those of the two isoforms. The other residues responsible for the difference in affinity have yet to be identified, although our results suggest that the unique tyrosine found near the C-terminus of thymosin β15 does not contribute to its actin-binding properties. Overall, our mutations confirm the importance of the β-thymosin C-terminus in its interaction with actin. Thus, the C-terminal variations commonly found in different members of the β-thymosin family may be critical determinants of the functional activity of each β-thymosin isoform.

Can the Higher Actin-Binding Affinity of Thymosin β15 Explain Its Ability to Regulate Cell Motility Independently?

We previously showed that thymosin β15 expression is essential for the enhanced motility of the highly motile prostate cancer cell lines that normally express this β-thymosin isoform. Cell motility depends on a tightly orchestrated collection of cellular processes including protrusion, contraction, adhesion and disadhesion, all of which involve actin filaments. Actin polymerization is tightly regulated in cells, and actin monomer sequestering proteins are critical to this regulation. Without these and other actin-binding proteins, greater than 99% of actin in cells would be in filaments [Pollard, 1986], which contrasts with the 20–50% filamentous actin normally observed. The affinity of the actin monomer sequestering protein and the kinetics of its interaction with actin are critical to current models of regulated actin polymerization [Carlier and Pantaloni, 1997].

Our studies as well as those of others demonstrate that the affinity of β-thymosin for actin is less than that of the high-affinity end of the actin filament. In the presence of free high-affinity actin filament ends, actin complexed to β-thymosin will dissociate and incorporate into the filament. In the presence of actin filaments that have their high affinity ends blocked with capping protein, β-thymosin will bind and sequester actin monomers, causing a net depolymerization of filaments.

Increased expression of higher-affinity actin monomer sequestering protein in cells in the presence of unchanged concentrations of actin and other actin-sequestering proteins will lead to an increase in the pool of sequestered monomers. This increase could enhance cell motility by at least two different mechanisms. Increasing either the monomer sequestering protein or the affinity of the monomer sequestering protein, or both, will drive the steady-state distribution of actin from filaments to monomers. This would lead to a net loss of F-actin, consistent with our observations of stress fiber loss in cells transiently expressing increased levels of β-thymosin. This decrease in F-actin correlates with a decrease in adhesion and an increase in motility in many cell types [Lauffenburger and Horwitz, 1996].

Concomitant with the net loss of F-actin there is an increase in sequestered actin available to polymerize upon the generation of a free high affinity filament end. The increased monomer concentration should lead to more extensive polymerization and to more protrusion as the two are intimately coupled. Therefore, increased expression of a higher-affinity actin monomer sequestering protein can both provide a greater pool of monomers for filament elongation and increase the turnover of filaments, leading to increased motility.

In summary, our results demonstrate that different members of the β-thymosin family can differ in their actin-binding and actin-sequestering properties. The truncation and hybrid data strongly suggest that the C-terminal amino acids contribute to actin-binding and are partly responsible for increased actin-binding of thymosin β15 relative to thymosin β4. This suggests that the C-terminal variations among β-thymosin isoforms may be important for the functional activity of these molecules in maintaining cytoskeletal architecture and dynamics. The various β-thymosins may have differential effects on properties including cell shape, cell movement, and cell polarity, among others that are mediated by the composition of their C-terminal domains.

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