J. Life Science. (2001), 11(1), 1~6

JOURNAL OF
Life Science
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Effect of Three Amino Acid Residues at the Carboxyl Terminus in Unacetylated α-Tropomyosin on Actin Affinity

Sun-Ju Jung, Sang-Min Seo, Kye-Hong Suh, Jae-Sub Yang and Young-Joon Cho†

Division of Life Science, Taegu University, Kyungsan, 712-714, Korea

Received: February 17, 2001

Abstract In order to determine the role of the carboxyl terminal amino acid residues of unacetylated α-tropomyosin in actin affinity two mutant tropomyosins were constructed by site-directed mutagenesis. TM16 was identical to the striated tropomyosin except that three amino acids in the carboxyl terminal end were altered to ^{282}TNM^{284} while in TM17 ^{282}TSI^{284} of the striated was replaced with ^{282}NSM^{284}. TM16 and TM17 were overproduced in *Escherichia coli* and analyzed for actin affinity by comparing actin affinities of the striated and TM11 (^{282}NNM^{284}). The apparent binding constants (Kapp) of unacetylated tropomyosins to actin were 5.1×10^4 M^{-1} for the striated, 1.1×10^5 M^{-1} for TM11, 1.09×10^5 M^{-1} for TM16, and 1.03×10^5 M^{-1} for TM17, respectively. Since the actin affinities of TM11, TM16, and TM17 were very similar, this result suggested that amino acid residues 282 and 283 were insignificant for actin affinity of unacetylated α-tropomyosin. However, they all exhibited higher actin affinities than that of the striated, suggesting that Met residue at the carboxyl terminus of unacetylated smooth tropomyosin was rather important for actin affinity, presumably due to the nucleophilic nature of sulfur atom in Met residue.

Key words: tropomyosin, actin affinity, carboxyl terminal, coiled-coil, unacetylated

Introduction

Tropomyosins are a family of proteins that is present in virtually all eukaryotic cells, all muscle cells and many of nonmuscle cells. It has high α-helical content and normally present in dimer. Ever since it was first discovered by Bailey [1], it has been considered as a model protein of α-helical coiled coil structure such as leucine zipper (for review, [2]). Muscle tropomyosins are fibrous molecule composed of two polypeptide chains, each (284 amino acid residues, 33,000 Da) in a two stranded coiled coil configuration. Two polypeptide chains are aligned in parallel and in register. Functions common to tropomyosins are to bind to F-actin cooperatively, to stabilize and stiffen to allow cooperative activation of the actin filament by myosin [3]. In striated muscle tropomyosin is in association with troponin complex to regulate interaction of actin and myosin in a calcium sensitive fashion. In smooth and nonmuscle cells tropomyosin may function as a modulator of microfilaments (for reviews, [4,5]).

Expression of different forms (isoforms) of tropomyosins in the cell is tissue-specifically and developmentally regulated and the tissue specific isoforms differ greatly in stability and functions such as their actin binding affinity, ability to regulate the actomyosin S1 ATPase, and polymerizability. These isoforms were generated either by alternative promoter from several genes or from a single gene by alternative splicing mechanism.

As a consequence of alternative splicing striated and smooth muscle α-tropomyosins differ only in exon 2 (amino acid residues 38-80) and exon 9 (residue 258-284) [6]. In the absence of troponin unacetylated striated tropomyosin bound poorly to actin whereas unacetylated smooth tropomyosin bound well to actin. It has been previously reported that the functional differences were attributed to the presence of tissue specific exon 9, which is located to the carboxyl terminus [7]. Hammell and Hitchcock-DeGregori [7] demonstrated that higher actin affinity of recombinant unacetylated smooth α-tropomyosin was ascribed to the carboxyl terminal nine amino acid residues among residues of the exon 9 since actin affinities of chimera sm9 and chimera TM9/a were indistinguishable.

In this experiment two recombinant unacetylated tropomyosins, TM16 and TM17, were constructed to define the role of the carboxyl terminal residues responsible for higher actin affinity. Two mutants were overexpressed in *E. coli* and purified and analyzed for actin binding. The results suggested that amino acid residues 282 and 283 had little effect on actin affinity of unacetylated α-tropomyosin. Rather residue 284 at the carboxyl terminus was significant for the higher actin

†Corresponding author
Phone: 82-53-850-6463, Fax: 82-53-850-6469
E-mail: yjcho@taegu.ac.kr.

affinity of unacetylated smooth α-tropomyosin.

Materials and Methods

Construction and expression of mutant recombinant tropomyosins

Rat striated (ST) α-tropomyosin cDNA was kindly provided by Dr. Nadal-Ginard (Harvard Medical School, Boston, MA). To construct mutant recombinant tropomyosin a double polymerase chain reaction was employed using *Pfu* DNA polymerase (Stratagene), which has 3' to 5' exonuclease activity for proofreading to minimize unintended mutagenesis [9]. PCR was carried out in a Hybaid Gradient PCR Express using pUC119/ST as a template. The first round of PCR was performed with 5' mutagenic sense primers (28 mers) whose sequences were shown in Table 1 and with M13/pUC reverse sequencing primer (19 mer) as a 3' antisense primer. The conditions for first round of PCR consisted of 94°C for 2 min followed by 20 cycles of 94°C for 90 sec, 55°C for 30 sec, 72°C for 1 min followed by a final step of 72°C for 7 min. After cleaning up the PCR product obtained from the first PCR with Wizard Prep Kit (Promega), it was used as a primer (megaprimer) for the second PCR. The second round of PCR was carried out, using pUC119/ST as a template, with 5'-primer (M13/pUC sequencing primer; 32mer) and 3'-megaprimer obtained from the first PCR. The amplification conditions comprise 94°C for 90 sec for denaturation followed by 30 cycles with 94°C for 90 sec, 64°C for 90 sec, and 68°C for 2 min and followed by final 7 min incubation at 72°C. After PCR steps the product was extracted and cleaned using a Wizard Prep Kit and was analyzed on agarose gel electrophoresis. The PCR products were digested with restriction enzymes *Nco*I and *Bam*HI. The resulting fragments were ligated to the *Nco*I and *Bam*HI sites of the expression vector pET11d (Novagen) and transformed into *E. coli* strain DH5α. The plasmids were isolated and were screened for mutants with appropriate restriction enzymes. The tropomyosin coding regions of the selected plasmids were then autosequenced to confirm the mutagenesis. The plasmids containing mutant TM DNAs, designated as pET 11d/TM16 and pET11d/TM17 were transformed into *E. coli* strain BL21 (DE3) and overexpressed for 4 hrs by the addition of isopropyl-β-thiogalactopyranoside (IPTG).

Purification of recombinant tropomyosin

Recombinant tropomyosins were isolated and purified as reported previously with minor modifications [10,11]. In brief bacterial cells were harvested after overproduction. The cells were lysed with lysozyme and freeze-thaw treatments followed by sonication in an IPG sonicator for 5 min with 0.5 power output, 50% duty cycle. Total lysate was centrifuged for 20 min at 18,000 rpm in a Sorvall SS34 rotor and 5 M NaCl was added to the supernatant to a final concentration of 1 M NaCl. The supernatant was then placed in a boiling water bath for 5 min and stood at room temperature for 1 hr to cool slowly. The heat-denatured proteins were removed by centrifugation at 15,000 rpm for 20 min in a SS34 rotor. The supernatant was precipitated with 35 to 70 percent ammonium sulfate and the pellet was dissolved in and dialyzed overnight against 20 mM Tris buffer, pH 7.0 containing 0.5 mM dithiothreitol (DTT). The dialysate was purified by DE52 (Whatman) DEAE-cellulose ion exchange chromatography. The column was equilibrated with the dialysis buffer and eluted with the same buffer containing a linear gradient of 0 to 1 M NaCl. Each fraction was analyzed on 12% SDS-polyacrylamide gel electrophoresis [12] and the fractions containing tropomyosin were further purified by BioGel HT (BioRad) hydroxyapatite column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl and 0.5 mM DTT and eluted with a linear phosphate gradient, from 10 mM to 200 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl and 0.5 mM DTT.

Chicken pectoral muscle actin was extracted from acetone powder, which was prepared from White Leghorn breast muscle and was purified as described [10,13].

Actin Binding Assay

Actin binding assay for tropomyosin was carried out by cosedimentation of proteins at room temperature as described [4]. In the actin binding assay tropomyosins were dialyzed separately overnight against a binding assay buffer which contains 10 mM imidazole buffer, pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, and 0.5 mM DTT. Following dialysis tropomyosins were prespun for 40 min at 25°C at 50,000 rpm in a TLA-100 rotor using Beckman Table Top Ultracentrifuge.

The conditions of the actin binding assay were described

Table 1. The amino acid sequences of the carboxyl terminal three amino acid residues and primer sequences

	282	283	284	mutagenic primer sequences
Striated (ST)	**T**	**S**	**I**	
TM11	**N**	**N**	**M**	5'-CAACGATATG**AACAACATG**TAAGTTTCT-3'
TM16	**T**	**N**	**M**	5'-CAACGATATGAC**TAACATG**TAAGTTTCT-3'
TM17	**N**	**S**	**M**	5'-CAACGATATG**AATTCAATG**TAAGTTTCT-3'
Smooth (sm9)	**N**	**N**	**M**	

TM11, TM16, and TM17 are identical to striated in sequence except the carboxyl terminal last three amino acid residues. Restriction sites introduced by mutagenesis for screening are underlined; both TM11 and TM16 were screened with *Afl*III and TM17 with *Eco*RI. Bold face letters represent codons for altered amino acids.

in the figure legends. Actin and tropomyosins were combined and vortexed extensively. The total volume of the mixture was 200 μl. To determine the total tropomyosin concentration 20 μl of aliquots was taken from the mixture prior to ultracentrifugation. 180 μl of the mixture was transferred to a ultracentrifuge tube and centrifuged under the same conditions mentioned above. Following centrifugation, the supernatant was transferred and the pellets was rinsed with 100 μl of the binding assay buffer and was resuspended in 35 μl of the same buffer. The supernatants and pellets were run on 12% SDS-polyacrylamide gel electrophoresis and the gels were stained with Coomassie Blue. For quantitative analysis of actin affinity the pellets and the supernatants were run on the 12% gels and stained with Coomassie Blue. Tropomyosin bands of the supernatants and actin and tropomyosin bands of the pellets were quantified by densitometry using a BioRad scanning densitometer Model GS-700. Binding constant (Kapp) and Hill coefficient (α^H) were estimated using SigmaPlot 2000 (SPSS) by fitting the data to the following equation.

$$v = n[TM]^{\alpha^H} \cdot Kapp^{\alpha^H} / 1 + [TM]^{\alpha^H} \cdot Kapp^{\alpha^H}$$

The data were normalized because the intensity of the staining was somewhat different from experiment to experiment.

Routine procedures for recombinant DNA techniques were carried out as described in methods manuals [14] or suggested by the suppliers or manufacturers. Protein concentrations were determined either by Bradford method [15] or by measuring absorbance at 280 nm. The extinction coefficients (1% at 280 nm) used were 11.1 for actin and 2.8 for tropomyosin, respectively. Oligonucleotide primers were synthesized from Bioneer and autosequencing was conducted using an ABI Prism Model 377 autosequencer at KAIST BioMedical Research Center in Taejon, Korea. All chemicals were reagent grade and purchased from Sigma. All restriction enzymes and DNA modifying enzymes were purchased from Boeringer-Mannheim unless specified in the text.

Results and Discussion

In order to define the role of three amino acid residues in the carboxyl terminal ends, two mutant tropomyosins were constructed by site-directed mutagenesis using PCR. As shown in Table 1, TM16 was identical to striated tropomyosin in amino acid sequence except that the carboxyl terminal three amino acid residues were substituted from TSI (Thr-Ser-Ile) of striated to TNM (Thr-Asn-Met). TM 17 was changed from TSI to NSM (Asn-Ser-Met). TM11 has NNM of smooth tropomyosin sequence instead of TSI of the striated. Mutant tropomyosins were constructed by site-directed mutagenesis employing a double polymerase chain reaction. After two rounds of amplification, the PCR product with an appropriate size of 1.3 kb long was digested with NcoI and BamHI and subsequently was cloned into pET11d expression vector. Each plasmid was screened with appropriate restriction enzymes for a new restriction site introduced as a result of mutagenesis (data not shown).

Bacteria harboring the plasmid were overexpressed by addition of IPTG and the large quantity of mutant tropomyosin was overproduced as shown in Figure 1. Upon lysis the vast majority of the protein was found in the soluble fraction (supernatant; *lane 2* in Panel A, Figure 1). Mutant tropomyosin was stable and remained in soluble fraction after heat treatment by boiling in water bath. Purification steps included ammonium sulfate fractionation, DE52 DEAE-cellulose ion exchange chromatography, and hydroxyapatite chromatography. Following purifications mutant TMs showed near to homogeneity, 95 to 99 percent as determined by densitometry (Panel B in Figure 1).

To assess the effect of three amino acids at the carboxyl terminus on actin affinity the binding isotherm experiment was performed with recombinant mutant tropomyosins to determine actin affinity quantitatively. As seen in Figure 2, all three TMs 11, 16, and 17 bound considerably stronger to actin than unacetylated striated TM while they bound weaker to actin as compared to unacetylated sm9. The apparent binding constants (Kapp) to actin were 5.1×10^4 M^{-1} for unacetylated striated (ST), 1.1×10^5 M^{-1} for TM11, 1.09×10^5 M^{-1} for TM16, and 1.03×10^5 M^{-1} for TM17, respectively as determined by curve-fitting with SigmaPlot 2000. Although TM 17 showed lower Hill coefficient indicative of cooperativity than TM11 and TM16, Kapp of TM11, 16, and 17 were comparable to each other and they bound stronger with approximately twice higher actin affinity than that of the striated. This result indicated that amino acid residue 282 and 283, at least exchange N to T or S, were insignificant for actin affinity since the difference in actin affinities of TM mutants with sequences of NNM, NSM, and TNM was negligible.

After this binding isotherm experiment was completed, it has been very recently reported that substitution of serine residue 283 with other amino acid residues significantly reduced the actin affinity [16]. The mutant bovine tropomyosin, Tm Ser283Glu that serine 283 was replaced with glutamate, showed substantially lower actin affinity than bovine Tm. Tm Ser283Ala and Tm Ser283Lys bound actin even more weakly than Ser283Glu. These results were in contrast with the result obtained from this experiment, suggesting that Ser 283 was insignificant in actin affinity. This apparent discrepancy may be due to the structural difference in amino terminus of tropomyosin molecule. The bovine Tm that Sano *et al.* [16] tested for actin binding assay was overproduced in insect cell with baculovirus expression system, in which generates mutant tropomyosin as N-acetyl form whereas bacterially produced mutant tropomyosin was unacetylated owing to the lack of N-acetylation machinery in *E. coli* [17]. Thus it was plausible to interpret that functional importance of

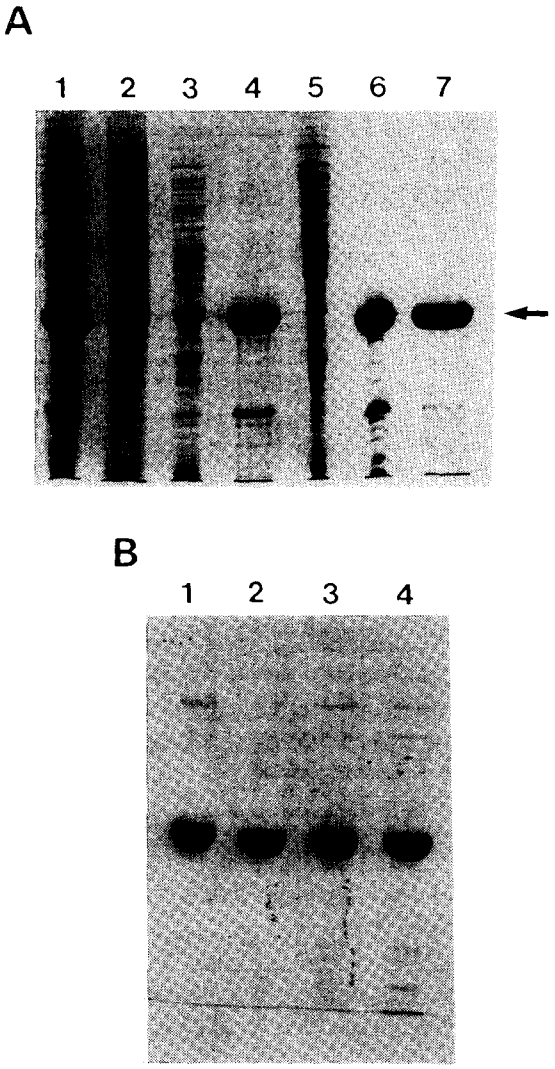

Fig. 1. SDS-polyacrylamide gel electrophoresis analysis of induction and purification of recombinant unacetylated tropomyosin After induction for 4 hrs, culture was harvested, lysed, and boiled as described in "Materials and Methods". 5 μl of aliquots from each step were loaded into 12% SDS-polyacrylamide gel. Unacetylated tropomyosin is marked with an arrow. Panel A. *Lane 1*, total lysate; *lane 2*, supernatant after lysis; *lane 3*, pellet after lysis; *lane 4*, supernatant after boiling; *lane 5*, pellet after boiling; *lane 6*, dialysate after 30-70 percent ammonium sulfate fractionation; *lane 7*, pooled fractions after DE52 DEAE-cellulose chromatography. Panel B. Unacetylated tropomyosins after hydroxyapatite chromatography were overloaded onto 12% SDS-polyacrylamide gel. *Lane 1*, TM16; *lane 2*, TM17; *lane 3*, TM11; *lane 4*, striated TM

serine 283 in actin affinity may be observed in N-acetylated form of tropomyosin and that serine 283 residue to some extent interact with amino terminal acetyl group as a consequence. Alternatively the effect of substitution of Ser to Asn in a mutant tested in this experiment may be neutral or silent in terms of actin affinity.

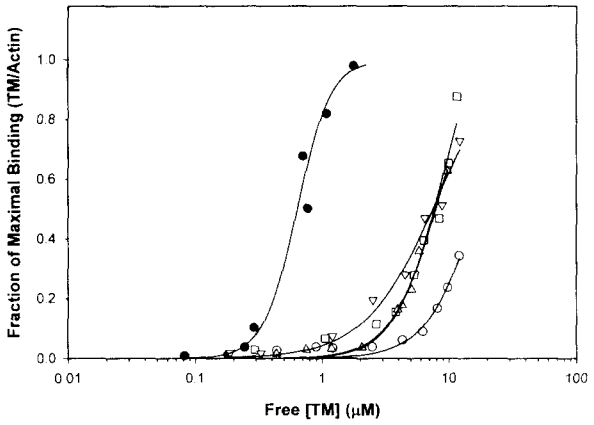

Fig. 2. Binding of unacetylated tropomyosin to actin. Unacetylated tropomyosins and actin were cosedimented as described under "Materials and Methods". The supernatants and pellets were analyzed by SDS-polyacrylamide gel. TMs in pellets (bound) and the supernatants (free) were quantified by densitometry. The data were fitted to the Hill equation using SigmaPlot 2000. Conditions: 5 μM actin, varied concentration of tropomyosin, in 10 mM imidazole, pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol. Symbols: ○, unacetylated striated; □, TM11; △, TM16; ▽: TM17; ●, unacetylated sm9

It has been reported that serine 283 of striated TM was phosphorylation site of tropomyosin molecule [18,19]. During embryonic development the phosphorylated form is predominant and increased level of phosphorylation has been observed in both embryonic skeletal and cardiac muscle. It was not clear whether unacetylated TM were phosphorylated or not. Unacetylated TMs were unlikely to be phosphorylated due to the facts that bacteria has no TM specific kinase and *E. coli* alkaline phosphatase readily dephosphorylates α-tropomyosin. Phosphorylation increases the ability of TM to polymerize alone and it also diminishes the enhancing effect which troponin has on this head-to-tail polymerization. Phosphorylation at serine 283, however, was not affected actin affinity since phosphorylated and nonphosphorylated tropomyosin showed very similar actin affinity [18,20]. Thus it was unable to determine whether unacetylated tropomyosins were phosphorylated unless measured the head-to-tail polymerization of tropomyosin molecules in the presence or absence of troponin.

The findings that amino acid residues 282 and 283 of unacetylated tropomyosin were insignificant in actin affinity and that TM 16 and 17 showed approximately two fold higher actin affinity than that of striated led to an implication that Met residue at the carboxyl terminus of smooth tropomyosin was mainly responsible for higher actin affinity. Furthermore a previous finding that unacetylated TM12 bound to actin very poorly and behaved like the unacetylated striated TM supported the importance of Met residue at the carboxyl terminus in smooth tropomyosin [11]. TM12 has the carboxyl terminal sequence of SGY (Ser-Gly-Tyr) from tmy-1 gene

of *Caenorhabditis elegans* and the rest of the sequence was identical to the striated [11]. This indicated that neither I nor Y but M has the effect on actin affinity.

Unlike muscle tropomyosin which the amino terminus was N-acetylated, the amino terminus of unacetylated tropomyosin may be protonated under the physiological and experimental conditions so that a positive charge was present at the amino terminus of the molecule. Unacetylated tropomyosin bound poorly to actin, was nonpolymerizable, and failed to regulate ATPase [10,17]. It had been proposed that the presence of a positive charge cause detrimental effect on actin affinity. Cho *et al.*, [10] proposed that the partial neutralization of a positive charge of the free α-amino group of unacetylated tropomyosin was insufficient to explain the large difference in actin affinity between acetylated and unacetylated forms but this explanation was not so straightforward. Cho *et al.*, [4] carried out the actin binding assay only qualitatively not quantitatively so that a deleterious effect of a positive charge at the amino terminus on actin affinity may not be excluded. Moreover although the pKa of the α-amino group of free methionine is 9.2, it is known that the pKa is influenced by the local environment, and one cannot be sure to what extent the amino terminus of the unacetylated tropomyosin molecule is deprotonated, unless the pKa is determined experimentally. An alternative explanation is that the unacetylated amino terminus destabilizes the α-helix at the amino terminus of tropomyosin by interfering with the helix dipole but the effect of a positive charge on stability of the helix dipole was controversial [21-24].

Based on the results from this experiment showing that TM17 exhibited higher actin affinity than the striated, it was suggested that Met residue at the carboxyl terminus had an effect on the actin affinity and that the Met might interact with protonated amino terminus of neighboring tropomyosin molecule. Since Met and Ile are both considered and classified as a nonpolar hydrophobic amino acid, it was surprising that the change between two conserved amino acids resulted in difference in actin affinity. Met (131.19 Da) is bigger than Ile (113.16 Da) in molecular weight but Ile is more hydrophobic and has only inert methylene and methyl group. However, Van der Waals volumes (124 $Å^3$) of these two amino acids are identical to each other so that they occupy similar space [25]. In contrast to Ile, Met has long unbranched nonpolar side chain and, in addition, Met contains sulfur atom. The sulfur atom is somewhat nucleophilic, but unlike other nucleophiles in proteins it cannot be protonated. Consequently, it is the most potent nucleophile at acidic pH in particular. Thus it was tempting to argue that the presence of the unshared electron pair of sulfur atom in Met at the carboxyl terminus as a nucleophile neutralized to some extent a positive charge on the amino terminus, thus alleviating any deleterious effect of a positive charge at the amino terminus on actin affinity by stabilizing interaction between the amino and carboxyl termini of tropomyosin molecules. This might imply that the carboxyl terminal Met be close contact with α-amino group of Met of neighboring tropomyosin molecule, which is consistent with nature of head to tail polymerization of tropomyosin molecules [26-28].

Previous results indicated that high actin affinity of smooth α-tropomyosin was attributed to the carboxyl terminal 9 amino acid residues [8]. Judged from the result from this experiment it was suggested that among 9 amino acid residues the high actin affinity may be attributed to the presence of 6 amino acids rather than 3 residues at the carboxyl terminus, since TM11, 16, and 17 showed significantly lower actin affinity as compared to sm9. Nonetheless they all have higher actin affinity than the striated so that they were in part responsible for higher actin affinity. Among the carboxyl terminal three amino acid residues Met at the carboxyl terminus was important for actin affinity, at least, for unacetylated tropomyosins while amino acid residues 282 and 283 were insignificant for actin affinity.

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