Glutamine Residue at 276 of smooth muscle α-tropomyosin is primarily responsible for higher actin affinity

Sun-Ju Jung and Young-Joon Cho*

Department of Genetic Engineering, Daegu University, Gyeongsan, 712-714

Previous reports indicated that the carboxyl terminal residues, glutamine276-threonine277 in particular, were important for actin affinity of the unacetylated smooth α-tropomyosin. To determine the role of the glutamine and threonine residues in C-terminal region in actin binding, we constructed mutant striated muscle α-tropomyosins (TMs), in which these two residues were individually substituted. These mutant tropomyosins, designated TM18 (HT) and TM19 (QA), were overexpressed in *E. coli* as an either unacetylated-form or Ala-Ser. (AS)-dipeptide fusion form, and were analyzed F-actin affinity by cosedimentation. Unacetylated TM19 (QA) bound to actin approximately three times stronger than TM18 (HT) and much stronger than ST (HA). AS/TM19 (QA) showed four times stronger in actin affinity than AS/ST (HA) while AS/TM14 (QT) bound to actin stronger to some extent than AS/TM18 (HT). These results suggested that the presence of Gln residue at 276 be primarily attributed to higher actin affinity of smooth α-tropomyosin.

Key words – recombinant tropomyosin, actin affinity, carboxyl terminal, overlapping region

Introduction

Tropomyosin (TM), which is an important regulatory protein in contractile system, is a highly conserved family of actin binding proteins and present in all types of muscle tissue and in many of nonmuscle cells [23,24,26 for reviews]. It has high α-helix content (over 90%) and normally present in dimer. Historically, the tropomyosins have been divided into two classes, high molecular weight and low molecular weight, which have 284 amino acid residues and 247 residues, respectively. High molecular weight tropomyosins are present in muscle cell whereas low molecular weight tropomyosins normally exist in nonmuscle cell [12,24]. Muscle tropomyosins are fibrous molecules composed of two polypeptide chains of 33,000 Da (284 amino acid residues) each in a two-stranded coiled coil configuration. Two polypeptide chains are aligned in parallel and in register. According to amino acid sequence analysis 40 heptapeptide repeats present in muscle tropomyosin molecule [18]. The heptapeptide (heptad) repeat (*abcdefg*) has 7 amino acid residues altering polar and nonpolar residues. Nonpolar amino acids occupy at *a* and *d* positions and these residues interface with nonpolar residues at *a* and *d* in the other strand. Hydrophobic interactions among the interfacing nonpolar residues primarily stabilize coiled-coil configuration of tropomyosin [9].

The function of tropomyosin in skeletal and cardiac muscle is in association with the troponin complex to regulate interaction of actin and myosin in a calcium sensitive manner. Tropomyosins bind cooperatively to F-actin along the long pitch grooves of the helical actin filaments [28]. They are required for actin filament stabilization and for cooperative regulation of many actin functions in most eukaryotic cells. Muscle tropomyosin spans the length of 7 actin monomers. Although periodic nature of actin binding site has been well established, both ends of tropomyosin molecule, the amino (N) and carboxyl (C) terminal regions may be more important for actin binding than periodic actin binding site repeats in tropomyosin molecule [20,21]. It has been revealed that local structures at the N-terminus greatly influence the actin affinity [4,5,14,27]. It has been also reported that carboxyl terminal amino acid residues define actin affinity of tropomyosin [2,3,13,15].

Mammalian and avian tropomyosins are encoded by four genes, α, β, γ, and δ. α- and β-tropomyosins have 284 amino acid residues and they are 85% identical to each other, whereas γ- and δ-tropomyosins are low molecular weight tropomyosins in nonmuscle cells [12,24]. Different forms of tropomyosins are present in many types of cells and they are characteristic of specific cell types and developmental stages. The tissue specific isoforms were gen-

*Corresponding author

Tel : +82-53-850-6463, Fax : +82-53-850-6469

E-mail : yjcho@daegu.ac.kr

erated from a single gene by alternative splicing as well as from the expression of different genes by alterative transcriptional promoter. As a consequence of alternative splicing, striated and smooth muscle α-tropomyosins differ only in exon 2 (amino acid residue 39-80) and exon 9 (amino acid residue 258-284) regions. It has been indicated that the functional differences, particularly in actin binding ability, were ascribed to exon 9 [2] and subsequently it has been reported that the carboxyl terminal 9 amino acids among 27 residues in exon 9 define actin affinity of tropomyosin [13]. Cho [3] reported that among the carboxyl terminal 9 residues glutamine276 and threonine277 were important for actin affinity.

In order to determine the carboxyl terminal amino acid residues that are responsible for actin affinity, we have constructed a series of mutants tropomyosins by oliogonucleotide site-directed mutagenesis method. TM18 (HT) and TM19 (QA) mutants were constructed from rat striated α-tropomyosin cDNA, in which the carboxyl terminal His276Ala277 of striated sequence α-tropomyosin were individually replaced with Thr at 277 and Gln at 276 of smooth α-tropomyosin, respectively. Unacetylated recombinant tropomyosin showed significant changes in tropomyosin function due to the alteration of the N-terminus, primarily to the lack of an acetyl group [5]. To examine actin affinity with recombinant tropomyosins functionally similar to muscle tropomyosin, we also constructed fusion tropomyosins with Ala-Ser dipeptide on the N-terminus of tropomyosin [19]. Recombinant mutant tropomyosins were overproduced in *E. coli* as unacetylated and Ala-Ser dipeptide fusion forms and actin affinities of TM18 (HT) and

Table 1. The carboxyl terminal 9 amino acids sequences of striated (ST), TM14 (QT), TM18 (HT), and TM19 (QA).

	276	277	278	279	280	281	282	283	284
striated(ST)	H	A	L	N	D	M	T	S	I
TM14 (QT)	Q	T	L	N	D	M	T	S	I
TM18 (HT)	H	T	L	N	D	M	T	S	I
TM19 (QA)	Q	A	L	N	D	M	T	S	I
heptad repeat	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*

TM14 was identical to striated sequence except that the amino acid residues 276-277 were substituted to QT of smooth. TM18, and TM19 have amino acid residue T at 277 and Q at 276 position, respectively, and the rest of the sequence is same as striated. The lower case letters *a* and *d* correspond to the interface residues in the heptapeptide (heptad) repeat of coiled coil structure.

TM19 (QA) were compared to those of ST (HA) and TM14 (QT).

Materials and Methods

Construction of mutant tropomyosins Recombinant mutant α-tropomyosins were constructed by polymerase chain reaction (PCR) with mutagenic oliogonucleotide primers. Expression plasmid pET11d/ST that contains rat striated α-tropomyosin cDNA (generous gift from Dr. Nadal-Ginard [25]) was used as a template. The PCR was carried out in a Hybaid PCR Express thermal cycler using *Pfu* DNA polymerase (Stratagene) which possesses 3' to 5' exonuclease activity. The PCR was performed with 5'-sense primer whose sequence was 5'-TAATACGACTCACTATAGGGGA ATTGTGAGC-3' (primer C; 31mer, T7 promoter primer) and 3' mutagenic antisense primer (63mer) whose sequences for TM18 (HT) and TM19 (QA) were 5'-TGGGAGGAGggat ccAAAGAAACTTATATGGAAGTCATATCGTTGAGA**GT GTGG**TCCAGCTCC-3' and 5'-TGGGAGGAGggatccAAAG AAACTTATATGGAAGTCATATCGTTGAG**CGCTT**GGTC CAGCTCC-3', respectively. The bold face letters correspond to mutagenic sites and the low case letters represent *BamHI* restriction site, which was introduced for removal of a long 3'-untranslated region of rat striated cDNA. The conditions for PCR were as follows; the template was denatured at 94°C for 2 min and 30 cycles of amplification at 94°C for 45 sec, 65°C for 60 sec and 72°C for 2min followed by 6 min at 72°C for a final extension. pET11d/TM14 was previously constructed by Cho [3] using a double polymerase chain reaction methods as described. Ala-Ser dipeptide fusion tropomyosins were constructed with 5' mutagenic primer whose sequence was CCACCGCCACC**ATGGCTA GC**ATGGACGCCATCAAG (35mer; the underlined corresponds to *NheI* site and mutagenic site is in bold face) and a 3' primer (primer D; 31mer, T7 terminator primer), respectively, using pET11d plasmids harboring corresponding cDNA sequences as templates. PCR conditions were as follows; 94°C for 2 min denaturation, 25 cycles of 94°C for 90sec, 60°C for 30sec, 72°C for 2min followed by a final step of 72°C for 6 min. The PCR products were cleaned using Wizard Prep Kit (Promega) as suggested by manufacturer and analyzed on agarose gel electrophoresis. DNA sequencing confirmed that no incidental changes had been introduced by PCR. The PCR products were cloned into *NcoI-BamH1* site in the expression plasmid pET11d and

transformed into *E. coli* strain BL21(DE3) and overexpressed for 4 hrs by addition of IPTG as previously described [3]. Primers were synthesized and purchased from Bionics. Autosequencing was carried out at Macrogen using an ABM Prism 3700.

Protein Purification Recombinant tropomyosins were isolated and purified as described previously [4]. All mutant tropomyosins were soluble upon lysis and they remained in stable and soluble form after heat treatment. Purification steps of mutant recombinant tropomyosins included ammonium sulfate fractionation, DE52 DEAE-cellulose ion exchange, followed by hydroxyapatite column chromatography. All mutant tropomyosins were successfully overproduced and purified to near homogeneity, over 95 percent as determined by scanning densitometry.

Chicken pectoral muscle actin was extracted from acetone powder, which was prepared from white Leghorn breast muscle and purified as described [3]. Protein concentration was determined by Bradford method [1] using bovine serum albumin as a standard or by measuring absorbance at 280 nm. The extinction coefficients (1% at 280 nm) used were 11.1 for actin and 2.8 for tropomyosins, respectively.

Actin binding assay Actin binding assay for tropomyosin was carried out by cosedimentation of F-actin and tropomyosins at room temperature in a Beckman Table Top Ultracentrifuge (Model TL-100) as described [3]. The conditions of the assay were as follows; 5 μM actin, 0 to 16 μM tropomyosins in 10 mM imidazole buffer (pH 7.0), 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT). The pellets (bound tropomyosins) and supernatants (free tropomyosins) were analyzed on 12% SDS-polyacrylamide gel electrophoresis [17]. Bands of actin and bound tropomyosin on the gel were quantified by densitometry using a Biorad scanning densitometer Model GS-700. Binding constants were estimated using SigmaPlot 2000 by fitting the data to the Hill equation as previously described [16]. The data were normalized because background values estimated by densitometer were to some extent different from gels to gels.

Results

Actin affinities of unacetylated tropomyosins

Actin binding assay was carried out by cosedimentation of F-actin with purified unacetylated tropomyosin. As an initial step we performed actin binding assay at certain concentration at 2 μM (at saturating concentration) of tropomyosin. As shown in Figure 1. TM14 (QT) and TM19 (QA) bound to actin well, whereas TM18 (HT) appeared to be bound poorly and ST (HA) hardly bound to actin. Subsequently we carried out the binding isotherm experiment to determine actin affinities quantitatively. The result was shown in Figure 2. All mutant tropomyosins (TM14,
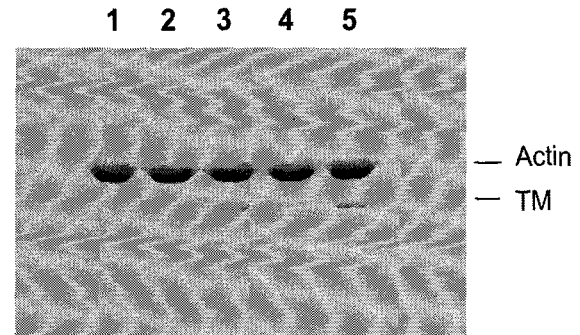

Fig. 1. SDS-polyacrylamide gel of pellets of actin binding assay with unacetylated tropomyosins. Binding of tropomyosins to actin was carried out by cosedimentation method in a Beckman Table Top Ultracentrifuge. Condition: 5 μM actin, 2 μM tropomyosin in 10 mM imidazole buffer, pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT. *Lane 1*, actin alone; *lane 2*, actin, unacetylated striated tropomyosin; *lane 3*, actin, unacetylated TM14; *lane 4*, actin, unacetylated TM18; *lane 5*, actin, unacetylated TM19.
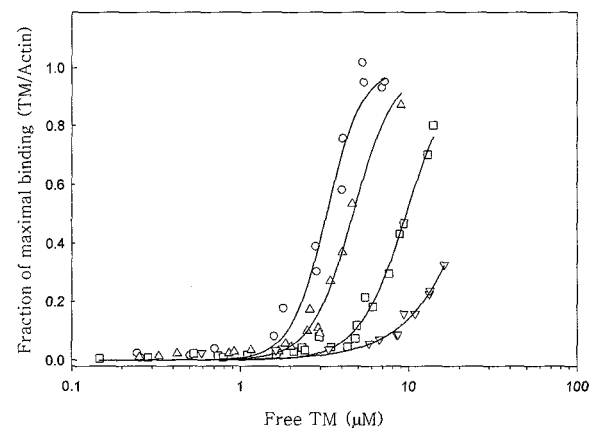

Fig. 2. Binding of unacetylated tropomyosins to actin. Condition: 5 μM actin, 0-16 μM tropomyosin, in 10 mM imidazole, pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT. The supernatants and pellets were analyzed by SDS-polyacrylamide gel. TMs in pellets (bound) and the supernatants (free) were quantified by densitometry and the data were fitted to the Hill equation using a SigmaPlot 2000. Symbols: ○, TM14 (QT); □, TM18 (HT); △, TM19 (QA); ▽, ST (HA).

18, 19) were showed higher actin affinities than that of the striated (ST). The fact that TM14 (QT) bound stronger than TM18 (HT) and TM19 (QA) was in consistent with previous result that smooth specific QT was responsible for higher actin affinity of unacetylated smooth tropomyosin. The apparent binding constants of unacetylated to actin were 0.14×10^5 M^{-1} for ST, 3.07×10^5 M^{-1} for TM14 (QT), 1.04 $\times 10^5$ M^{-1} for TM18 (HT), 2.15×10^5 M^{-1} for TM19 (QA), respectively. The results that TM19 (QA) bound much stronger, at least 10 times, than ST (HA) and that TM14 (QT) bound to actin approximately three times stronger than TM18 (HT) indicated that between Gln and Thr residues of TM14 (QT) the Gln at 276 was attributed more to higher actin affinity than Thr at 277 in unacetylated tropomyosins. However, Thr was in some extent important since TM14 (QT) showed higher actin affinity than TM19 (QA). Thus these result strongly suggested that both Gln and Thr were required for fully high-actin affinity of smooth tropomyosin and that between Gln and Thr residues the Gln was more important for actin affinity of unacetylated tropomyosins.

Actin binding of Ala Ser (AS) dipeptide fusion tropomyosin

Unlike muscle tropomyosin whose N-terminus was blocked by acetyl group, recombinant tropomyosins were unacetylated as produced in bacteria. Unacetylated tropomyosins bound poorly to actin and were nonpolymerizable, and were unable to regulated ATPase activity in the absence of troponin [5]. The addition of Ala Ser (AS) dipeptide to the N-terminus of unacetylated tropomyosin restored functions of muscle tropomyosin and appears that AS dipeptide extension was sufficient for role of the acetyl group. As seen in Figure 3, all AS/TMs were showed higher actin affinity than unacetylated tropomyosins. The apparent binding constants (summarized in Table II) to actin were 8.63×10^6 M^{-1} for AS/ST (HA), 10.75×10^6 M^{-1} for AS/TM14 (QT), 8.61×10^6 M^{-1} for AS/TM18 (HT), and 35.5×10^6 M^{-1} for AS/TM19 (QA), respectively. Actin affinity of AS/TM19 (QA) was three to four folds higher than those of AS/TM14 (QT), AS/TM19 (QA), and AS/ST (HA), which were comparable to each other. This result indicated Gln was more important residue than Thr for higher actin affinity of smooth tropomyosin as exhibited with unacetylated tropomyosin. In addition AS/TM14 (QT) showed higher actin affinity than AS/TM18 (HT) and
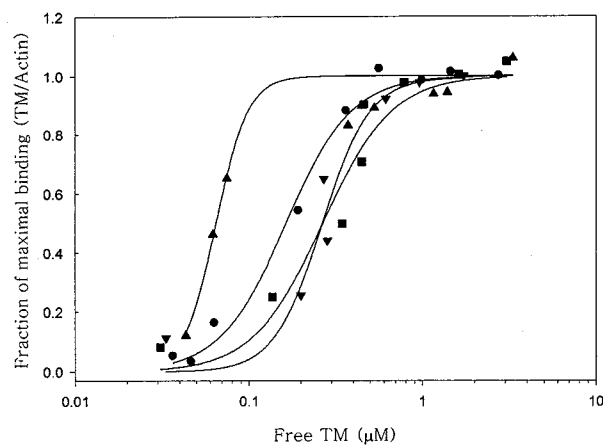

Fig. 3. Binding of Ala-Ser dipeptide fusion tropomyosins (AS/TMs) to actin. Condition: 5 μM actin, 0-3 μM tropomyosin, in 10 mM imidazole, pH 7.0, 150 mM NaCl, 2 mM MgCl$_2$, 0.5 mM DTT. Symbols: ●, AS/TM14 (QT); ■, AS/TM18 (HT); ▲, AS/TM19 (QA); ▼, AS/ST (HA).

Table 2. Actin binding constants and Hill coefficients of recombinant tropomyosins

	Unacetylated		Ala-Ser dipeptide fusion	
	$K_{app}(10^6$ M$^{-1})$	α^H	$K_{app}(10^6$ M$^{-1})$	α^H
Striated	≪0.1	N.D.	8.6±0.9	3.1±1.4
TM14	0.31±0.02	4.1±0.9	10.8±0.9	2.3±0.3
TM18	0.10±0.01	3.1±0.3	8.6±1.5	2.2±0.7
TM19	0.21±0.01	3.5±0.4	35.5±2.3	4.7±1.8

The data from the binding isotherms shown in figure 1 and 2 were fitted to the Hill equation. Data were reported as K_{app} (apparent binding constant) and α^H (Hill coefficient) ± Standard Error calculated using SigmaPlot 2000(SPSS).

AS/ST (HA) and this result also supported importance of Gln at 276 for actin affinity. Since AS/TM18 (HT) and AS/ST (HA) showed similar actin affinity, the presence of Thr or Ala at 277 appeared to be insignificant for actin affinity in AS/TMs. Nevertheless, unlike unacetylated tropomyosins, AS/TM19 (QA) exhibited significantly higher actin affinity than AS/TM14 (QT) and this result suggested that Ala residue may be more important for actin affinity of AS/TM than Thr.

Discussion

The carboxyl terminal region of α-tropomyosin is encoded by tissue specific exon 9, which corresponds to 253-284 amino acid residues. This region of α-tropomyosin contains troponin T binding site and interacts with the

amino terminal region of a neighboring tropomyosin with head-to-tail fashion. The entire carboxyl terminal region is required for troponin to promote fully high-actin affinity. Among residues 253 to 284 first 18 residues are necessary for the interaction of troponin with tropomyosin, 9 amino acids from the carboxyl terminal end define actin affinity [5,13]. In this study aimed to determine a residue(s) responsible for high actin affinity of tropomyosin, our results, together with previous findings from our laboratory, showed that among the carboxyl terminal 9 residues the Gln276 is most important for actin affinity of unacetylated and AS dipeptide fusion tropomyosin.

The Gln276 residue is located at c position of heptapeptide repeat and at the external face of the helices, and it may be directly involved interaction to actin. It has been thought that the carboxyl terminal end of tropomyosin molecules form overlap region with the amino terminal end, which is comprised of about 9 to 11 amino acids from each end. As a result, Gln276 may alter interactions of residues within the overlap region and affect stability of the overlap region, and in turn indirectly influences on actin affinity. The three dimensional NMR studies revealed that the carboxyl terminal region residues 270-279 of tropomyosin was flexible and did not form a coil-coil while residues 253-269 form a canonical coiled coil in solution [8,10]. It has been suggested that flexibility of tropomyosin be essential for its end-to-end interactions and regulatory functions. Accordingly it may be interpreted that the presence of Gln at 276 resulted in higher actin affinity of α-tropomyosin due to the increased conformational flexibility of the overlap region.

It is interesting that effect of residue at 277 on actin affinity differs in unacetylated and AS tropomyosins. McLachlan and Stewart [18] proposed a model showing structure of the overlap region. Palm et al. [21,22] and subsequently Greenfield et al. [10] reported a helical wheel model of possible overlap interaction. According to these models, the C-terminal Ala277 and Ile284 may be involved in hydrophobic interaction with the N-terminal Met1 and Met8, respectively. Due to the lack of acetyl group on the N-terminus, Met1 in unacetylated tropomyosin expected to be positively charged under the condition that we tested. The presence of a positive charge on the N-terminus may interfere with hydrophobic interaction between Ala277 and Met1. Consequently polar residue Thr, rather than non-polar Ala, at 277 may be favored in unacetylated tropo-

myosin, which implies the role of Thr277 in actin affinity of unacetylated tropomyosins. On the other hand Met1 of Ala-Ser dipeptide fusion (AS) tropomyosin may form hydrophobic interaction with Ala277. The presence of Thr instead of Ala at 277 may interrupt hydrophobic interaction with Met1, which may explain a finding that AS/TM19 (QA) bound stronger than AS/TM14 (QT).

Mammalian α- and β-striated muscle tropomyosin differ in residue at 276, where histidine and asparagine residues occupy, respectively. Gaffin et al. generated transgenic mouse line, α-TMHis276Asn, in which His residue at 276 of α-tropomyosin was replaced with Asn residue of β -tropomyosin [6,7]. They demonstrate that a charge change at the carboxyl terminal region of α-tropomyosin alter the functional characteristics of cardiac muscle contractility. The α-TMHis276Asn fiber produced more force per given $[Ca^{2+}]_{IC}$ when compared to non-transgenic fibers. Asn and Gln residues are in general considered to be comparable in nature. Judging from our results showing that the presence of Gln at 276 was contributed to higher actin affinity, the increase in force generation in muscle fiber from transgenic mice may be due to the increase in actin affinity, implying functional importance of Gln residue at 276 of tropomyosin.

Acknowledgement

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초록 : 평활근 α-트로포마이오신 Gln276잔기의 액틴친화력에 대한 중요성

정선주 · 조영준*

(대구대학교 자연과학대학 유전공학과)

평활근 α-트로포마이오신의 높은 액틴 친화력은 아미노산 잔기 Gln276 및 Thr277에 기인한다는 이전 보고에 따라, 2 잔기 중 어느 잔기가 액틴 친화력에 더 중요한 가를 알아보기 위하여 골격근 트로포마이오신의 His 혹은 Ala 단일 잔기를 각각 Gln 혹은 Thr으로 치환한 돌연변이 트로포마이오신을 제작하여 대장균에서 대량발현 시킨 후 정제하여 액틴 결합력을 측정하였다. 비록 비아세틸화된 트로포마이오신의 경우 Gln 및 Thr 잔기가 최고 액틴 친화력을 위해 모두 필요하나, 돌연변이 트로포마이신 중 Gln 잔기를 가진 돌연변이 트로포마이오신들이 다른 돌연변이 트로포마오신들에 비하여 3에서 4배 높은 액틴친화력을 보였다. 이러한 결과는 평활근 α-트로포마이오 신의 높은 액틴 친화력은 Thr277 잔기보다 Gln276 잔기에 주로 기인한다는 것을 의미한다.