Overexpression of sTnC polypeptide in muscle cells is controlled by its rapid degradation

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Abstract The check-points that maintain stoichiometric synthesis of muscle proteins were examined by misexpression of slow troponin C (sTnC) in mouse C2 myotubes. The sTnC mRNA was overexpressed in myotubes by transfecting these cells with a plasmid construct containing the constitutive CMV promoter-driven sTnC cDNA. An approximately four-fold increase of sTnC mRNA level in the transfected cells was observed. However, the increased mRNA level did not produce a corresponding increase of the sTnC polypeptide level in transfected cells. Only a modest 1.5-fold increase of the sTnC polypeptide level in the transfected cells was observed. The excess sTnC polypeptide in transfected cells was found in the soluble form which was not complexed with other thin filament proteins. The difference between the increase of sTnC mRNA and the polypeptide levels in transfected cells was not due to inefficient translation of the overexpressed sTnC mRNA. Analyses of the stability of the sTnC polypeptide in the thin filament and in the unassembled soluble forms showed that the excess soluble sTnC polypeptide was degraded more rapidly than the sTnC polypeptide of the thin filament. Analyses of the mRNA and polypeptide levels of several thin filament complements showed no effect of overexpression of the sTnC mRNA.

Key words: Muscle protein; Troponin C; Overexpression; Protein degradation

1. Introduction

Formation of the contractile apparatus in muscle cells requires the activation of several genes and proper assembly of their polypeptide products. During differentiation of committed myoblasts to terminally differentiated myotubes expressions of various muscle specific genes are coordinately activated [1,2]. Most of the contractile proteins are coded by single copy genes [1], yet different amounts of these proteins are produced from these genes to form the myofilament. For instance, seven molecules of actin and one molecule of each of the troponin subunits and tropomyosin (Tm) are required to form the thin filament [3].

Differences in the rates of transcription among the genes for contractile proteins may produce different amounts of mRNA from these genes. Also differences in mRNA stability, efficiency of their translation and stability of polypeptides, all contribute toward the final steady state level of polypeptides [4,5]. How all of these levels of regulation are fine tuned to produce stoichiometric amounts of the different contractile proteins to form the myofilament is not clear. Misexpression of one component of the myofilament will disrupt the stoichiometry. How this process might influence the assembly and maintenance of the myofilament and affect the synthesis of other contractile proteins needs to be examined in detail.

Various methods may be used for misexpression of a polypeptide partner of the myofilament. One simple method is to use antisense oligodeoxynucleotides to block the expression of a specific gene for one of the muscle proteins [6–11]. Another approach is the transient overexpression of a specific polypeptide of the myofilament by transfecting muscle cells with an appropriate ectopic gene. We chose slow troponin C (sTnC) as a model system to investigate the effect of its misexpression in muscle cells. Troponin C is the smallest subunit of the troponin complex involved in regulating muscle contraction by Ca^{2+} [3,12]. Troponin C is a highly conserved Ca^{2+} binding protein [12]. The two known isoforms of TnC (slow and fast) are separate gene products [13,14]. The fast TnC occurs exclusively in fast skeletal muscle, whereas the slow isoform is present in both skeletal and cardiac muscle. However, in cardiac muscle the slow isoform is the only isoform found in the troponin complex [14–16]. Generally the TnC genes are not expressed in non-muscle cells. The only known exceptions are the presence of sTnC in human fibroblasts and chicken liver and brain cells [17,18]. The C2 myogenic cell line derived from mouse skeletal muscle following differentiation to myotubes produces predominantly the slow TnC isoform as well as a small amount of the fast isoform [13,14]. The expression pattern of sTnC in C2 muscle cells is less complicated than other muscle proteins which exhibit a more complex isoform expression pattern in muscle cells [19]. Therefore, misexpression of the predominant isoform of TnC in these cells may be a good model to study the check-points that control stoichiometric production of contractile proteins.

In previous studies, misexpression of the sTnC gene was achieved by blocking its expression by antisense oligodeoxynucleotides. Although 50–70% inhibition of synthesis of these polypeptides was achieved, very little effect was seen on the synthesis of other contractile proteins [6–8]. Detailed investigation of how the myofilament is maintained under reduced sTnC synthesis revealed that the stability of the sTnC polypeptide is increased to compensate for its reduced synthesis

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Abbreviations: sTnC, slow/cardiac troponin C; TnT, troponin T; Tm, tropomyosin; TnI, troponin I

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[8]. The studies reported here used overexpression of sTnC to disrupt the stoichiometric synthesis of contractile proteins. The results show that the proper myofilament assembly was maintained by rapidly degrading the unassembled excess sTnC.

2. Materials and methods

2.1. Construction of sTnC expression vector

A full length mouse sTnC cDNA clone was obtained from IMAGE consortium (image 1139312). The entire cDNA insert was amplified by PCR using the d(5′-ggatccGGGAGCAAGGATTTATGTG) and 5′-cctaggTTCGGATCCTTGGTGAGCT primers with recognition sequences of *Bam*HI and *Avr*II restriction enzymes respectively (indicated by lowercase letters). The PCR reaction was carried out in the buffer containing 10 mM Tris–HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 μM dNTPs, 200 ng of each primer, 2 units of Taq polymerase, 20 ng of image sTnC cDNA clone as template for 30 cycles [20]. Each cycle was at 94°C for 15 s, 50°C for 30 s and 72°C for 1 min and the final cycle was at 72°C for 5 min. The amplified product was purified by ion exchange chromatography using a PCR purification kit (Qiagen, Valencia, CA, USA) and digested with *Avr*II and *Bam*HI restriction enzymes. The digested product was gel purified using a Qiagen kit. For constructing the sTnC expression vector, this fragment was ligated to the 4.3 kb *Avr*II/*Bam*HI fragment of CMV-SPORT-β-gal plasmid (Gibco/BRL, Burlington, ON, Canada).

2.2. Cell culture

Mouse skeletal muscle C2 myoblast cell line (ATCC CRL 1772) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada). Differentiation of myoblasts to myotubes was induced by transferring 60% confluent cells to the serum free OPTIMEM (Invitrogen) for 24 h followed by addition of 2.5% horse serum containing DMEM. Long multinucleated myotubes were formed after 3 days of induction of differentiation. The mRNA for contractile proteins appeared within 12 h of induction [21].

2.3. Transfection of cells

Approximately 5×10^5 C2 cells grown on a six-well dish were transfected with the appropriate plasmid DNA, after transfer to the OPTIMEM medium for differentiation. 1–2 μg of DNA was mixed with 10 μg of lipofectamine (Invitrogen) in 400 μl of OPTIMEM at 20°C for 30 min before being added to the cells. Cells were incubated with the DNA/liposome mixture for 24 h, and then the medium was removed, and cells were allowed to differentiate in 2.5% horse serum containing DMEM for 2 days [20,21].

2.4. Measurement of mRNA levels

Cells were washed three times with phosphate buffered saline (PBS, 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.2) and total cellular RNA was isolated according to the manufacturer's protocol using the high pure RNA isolation kit (Roche Biochemicals, Laval, QC, Canada). The quality of the RNA sample was determined by 1% agarose gel electrophoresis [22] and samples containing undegraded ribosomal RNAs were used for further analysis. The levels of different mRNAs were measured by S1 nuclease protection of the appropriate antisense oligodeoxynucleotide probe. The oligomer probes used for the analysis of sTnC, f-troponin T (fTnT), f-troponin I (fTnI), α-Tm and α-acting mRNAs were d(5′-ATCCGACAGCTCCTCCTCAGACTTCCCTTT), d(5′-TCAAAGTGGCTGTCAATGAGGGCTTGGAGC), d(5′-CCTTGCTGCTCTTCTGCACCTTCACCTCCA), d(5′-TCTTTCAGCTGGATCTCCTGAATCTCCATC) and d(5′-TGAGGGTGACACCGTCCCCAGAATCCAACA) respectively. Oligomers were 5′ end labeled with [γ^{32}P]ATP and T4 polynucleotide kinase. Nuclease protection was performed with 5 μg of total cellular RNA and 200 ng of radiolabeled probe using the multi-NPA kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The S1 nuclease protected probe product was analyzed by 8 M urea–20% polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. The nuclease protected probe was quantified by scanning the autoradiogram using the Bio-Rad scanner.

2.5. Sucrose gradient centrifugation

Approximately 5×10^6 cells were lysed in 800 μl of a lysis buffer (25 mM Tris–HCl, pH 7.6, 250 mM KCl, 10 mM $MgCl_2$, 0.5% Nonidet P-40, 200 μg/ml heparin, 50 μg/ml cycloheximide) and centrifuged at $12\,000\times g$ for 10 min. The $12\,000\times g$ supernatant fraction was centrifuged in a 10 ml 10–50% sucrose gradient containing the lysis buffer at 40 000 rpm in a Beckman SW41Ti rotor for 2.5 h [20]. The gradient was fractionated using a Buchler Auto Densi-Flow IIC apparatus. Total RNA from each fraction was isolated by using 4 M guanidine thiocyanate as previously described [23] and precipitated with ethanol using 5 μg of yeast tRNA as the carrier.

2.6. Measurement of protein levels

Cellular levels of specific contractile proteins were measured by immunoblotting. The cells were washed three times with phosphate buffered saline (PBS, 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.2) and lysed in 200 μl of a buffer containing 25 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10 mM dithiothreitol (DTT), 10% glycerol, and 0.01% bromophenol blue. The cell lysate was subjected to 12.5% SDS–PAGE as described previously [24] and the separated polypeptides were transferred to a nitrocellulose membrane. The levels of different polypeptides were determined by using appropriate antibodies [8]. The antigen–antibody complex was visualized by horseradish peroxidase conjugated (POD) anti-rabbit antibodies using the Lumi-Light Western blotting kit (Roche, Laval, QC, Canada) according to the manufacturer's protocol.

2.7. Measurement of thin filament associated TnC levels

The presence of TnC polypeptide in the thin filament complex was measured by immunoprecipitating the troponin complex using the TnT antibody (Sigma, St. Louis, MO, USA). Cells were lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) and pre-incubated with an equal packed volume of protein A-Sepharose beads (Sigma) for 1 h. The precleared supernatant of the cell lysate obtained after 1 min centrifugation at $12\,000\times g$ was incubated with the TnT antibody for 16 h at 4°C [25]. Protein antibody complexes were then incubated with an equal packed volume of protein A-Sepharose beads for 2 h. The beads were then washed four times in RIPA buffer and the bound proteins were eluted at 95°C in the sample buffer (25 mM Tris–HCl, pH 6.8, 2% SDS, 10 mM DTT, 10% glycerol and 0.01% bromophenol blue) for SDS–PAGE. The eluted samples were analyzed by 12.5% SDS–PAGE. The co-precipitation of TnC with TnT was examined by immunoblotting using sTnC antibody as described above.

3. Results

3.1. Misexpression of TnC

To investigate how an imbalance in the stoichiometry of the thin filament proteins affects the regulation of expression of the contractile protein genes we have transfected differentiating cells with a construct containing the CMV promoter-driven mouse sTnC cDNA. Transfection efficiency of myotubes was poor, therefore, we have transfected the myoblasts immediately after they were transferred to the differentiation medium. Co-transfection of these cells with β-galactosidase expression vector CMV-SPORT-β-gal (Invitrogen) showed that approximately 30% of cells were transfected. The level of mRNAs for sTnC, α-actin, fTnT, fTnI and α-Tm in myoblasts, myotubes and mock-transfected cells were measured by S1 nuclease protection analyses. The results show that the level of sTnC mRNA in transfected cells was approximately four-fold higher than that in the mock-transfected cells (Fig. 1). The levels of mRNAs for other thin filament proteins, α-actin, α-Tm, fTnI and fTnT were not influenced by the increased level of sTnC mRNA in transfected cells. The mock-transfected cells and the cells transfected with the sTnC cDNA construct both showed comparable levels of α-actin, α-Tm, fTnI and fTnT mRNAs. None of these mRNAs includ-

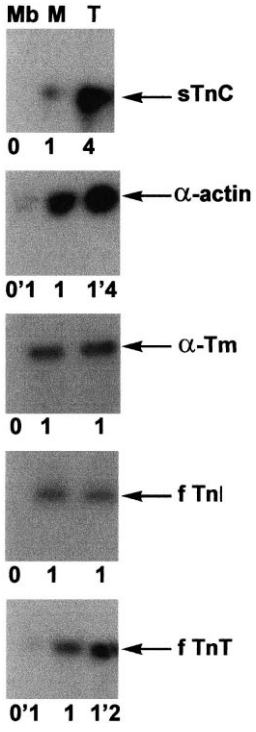

Fig. 1. Levels of different mRNAs coding for the thin filament proteins. The steady state level of different mRNAs from myoblast (Mb), mock-transfected (M) and transfected cells (T) was measured by S1 nuclease protection of oligodeoxynucleotide probes as described in Section 2. The autoradiograms were quantified by using a Bio-Rad gel scanner. The relative levels of mRNAs are shown under each panel. The positions of different mRNAs are shown by an arrow beside each panel.

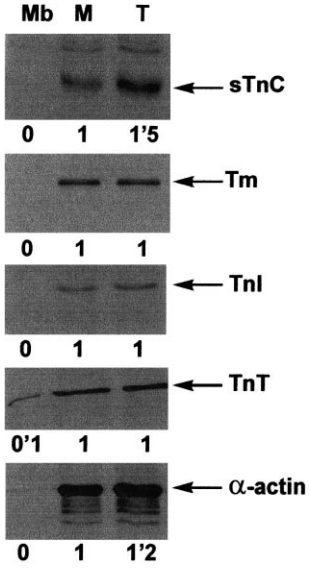

Fig. 2. Levels of different polypeptides of the thin filament. The polypeptide levels of thin filament complements in myoblast (Mb), mock-transfected (M) and transfected cells (T) were measured by immunoblotting using appropriate antibodies as described in Section 2. The immunoblots were scanned by using a Bio-Rad gel scanner. The relative levels of different polypeptides are shown at the bottom of each panel. The positions of different polypeptides are shown by arrows beside each panel.

ing the sTnC were present in significant amounts in the proliferating myoblasts (Fig. 1).

To further examine whether overexpression of the sTnC mRNA resulted in over-production of the corresponding polypeptide, the level of sTnC polypeptide was measured by immunoblotting. Results show that the sTnC level in transfected cells was only approximately 1.5-fold higher than that in the mock-transfected cells (Fig. 2). Analyses of the α-actin, fTnT, fTnI and α-Tm levels showed no difference in their levels between transfected and mock-transfected cells (Fig. 2). Very little or no detectable levels of the thin filament proteins were found in proliferating myoblasts (Fig. 2, Mb). These results therefore suggest that a nearly four-fold overexpression of sTnC mRNA did not result in a proportional increase in the sTnC polypeptide level.

3.2. Translation of sTnC mRNA

To test whether the sTnC mRNA was poorly translated in the transfected cells, we examined the distribution of cytoplasmic sTnC mRNA in a sucrose gradient. Analysis of the absorption profile of a representative gradient at 260 nm showed that fractions 3–5 contain ribosomal subunits and monosomes while fractions 6–12 contain polyribosomes. Results of sTnC mRNA distribution in the sucrose gradient show that most of

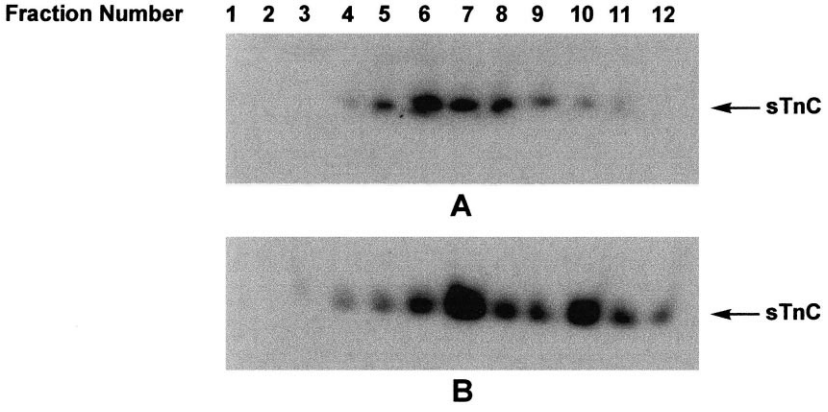

Fig. 3. Cytoplasmic distribution of sTnC mRNA. The cells were lysed in a polysome buffer and the 12 000×*g* supernatant fraction was centrifuged in a 12 ml 10–50% sucrose gradient at 40 000 rpm for 2.5 h in a Beckman SW41Ti rotor as described in Section 2. 12 fractions were collected and RNA from each fraction was isolated for analysis of sTnC mRNA level by S1 nuclease protection of the probe as described in Section 2. A: Mock-transfected myotubes after 3 days of treatment without plasmid DNA. B: Myotubes after 3 days of transfection.

the sTnC mRNA in both mock-transfected (Fig. 3A) and transfected cells (Fig. 3B) was present in the translated polyribosomal fractions (fractions 6–12). However, the translated sTnC mRNA of transfected cells showed a bimodal distribution (Fig. 3B). This was probably due to a difference in size between the endogenous and the ectopic sTnC mRNAs since short vector derived sequences were present at both 3′ and 5′ untranslated regions of the ectopic mRNA. Approximately 15% of the total cytoplasmic population of the sTnC mRNA in both mock-transfected and transfected cells was present in the non-translated region of the gradient (fractions 1–5). Therefore, no difference in the translation of sTnC mRNA between the transfected and mock-transfected cells was found. The relatively poor (1.5-fold) overexpression of the sTnC polypeptide in spite of the nearly four-fold increase in the sTnC mRNA level in cells transfected with the sTnC expression vector was not due to translational repression of the sTnC mRNA.

3.3. Stability of sTnC

Since the results of our studies discussed above showed that the level of sTnC was less than what was expected for the overexpressed translationally active sTnC mRNA, we examined the possibility of a decrease in the stability of the sTnC polypeptide in transfected cells. For these studies we first examined the distribution of sTnC between the troponin complex and free cytoplasmic states. The troponin C was co-immunoprecipitated with an antibody to another polypeptide partner, the TnT, of the troponin complex of the thin filament. The complex bound to protein A-Sepharose and the unbound fraction were both analyzed by immunoblotting for the presence of sTnC. The results (Fig. 4) show that nearly all of the sTnC was complexed with TnT (Fig. 4A) in mock-transfected cells. However, in the transfected cells, approximately the same amount as that of the mock-transfected cells was present as troponin complex. But nearly 50% of the sTnC was not present as the troponin complex of the thin filament (Fig. 4B). No detectable level of sTnC was found in the myoblasts (Fig. 4, Mb).

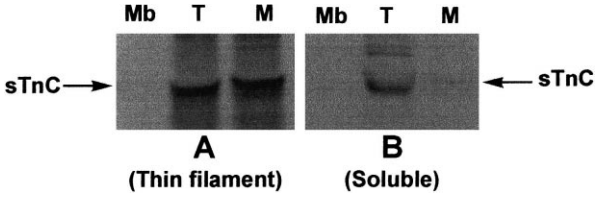

Fig. 4. Analysis of the presence of sTnC in troponin complex. Cell lysates were prepared in RIPA buffer as described in Section 2. Samples of cell extracts containing equal amounts of protein were precleared with an equal packed volume of protein A-Sepharose (Sigma) for 1 h. The precleared cell extract was incubated with TnT antibody (Sigma) for 16 h at 4°C [9]. The antigen–antibody complex was then further incubated with an equal packed volume of protein A-Sepharose for 3 h at 4°C. The Sepharose beads were washed in RIPA buffer and the bound proteins were eluted with an SDS containing buffer. The eluted polypeptides were separated by PAGE. The protein A-Sepharose unbound fractions were also separated by PAGE. The presence of sTnC in both eluted and unbound fractions was analyzed by Western blotting using sTnC antibody (Research Genetics) as described in Section 2. Protein A-Sepharose bound (A) and unbound (B) fractions; M, mock-transfected cells; Mb, proliferating myoblasts; T, transfected cells.

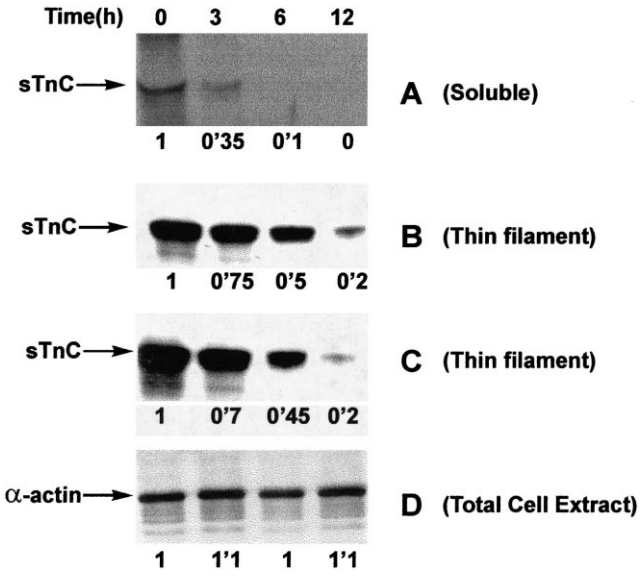

Fig. 5. Stability of polypeptides. Cells were treated with 50 μg/ml cycloheximide for different times and the level of thin filament bound and soluble sTnC was analyzed as described in the legend to Fig. 4. As control the level of α-actin was also analyzed in the total cell extract. The immunoblots were performed with sTnC (A–C) and α-actin antibody (D). The intensities of sTnC and α-actin bands in these blots were determined by scanning using a Bio-Rad scanner and were shown at the bottom of each panel. A: Soluble sTnC in transfected cells. B: Thin filament associated sTnC in transfected cells. C: Thin filament associated sTnC in mock-transfected cells. D: α-actin in the total cell extract of transfected cells.

Since the excess sTnC was not present in the thin filament complex in the transfected cells, we then examined the stability of sTnC in the free and thin filament bound forms. For these analyses, cells were treated with cycloheximide to inhibit protein synthesis and the level of sTnC in the troponin complex and free state was determined by immunoblotting at different times after the drug treatment as described above. The results of immunoblotting to determine the sTnC levels are shown in Fig. 5. The results show that the thin filament bound sTnC was degraded with a half life of approximately 6 h in both mock-transfected and transfected cells. The non-stoichiometric excess sTnC was present mostly in the cytoplasmic free form (Figs. 4 and 5) and was more rapidly degraded than the thin filament bound form. The half life of free sTnC of transfected cells was only 3 h. As a control, we have also examined the stability of α-actin levels in transfected and mock-transfected cells. During the time course of our studies the α-actin levels showed no detectable change suggesting that the α-actin is considerably more stable than the sTnC.

4. Discussion

Most proteins function as multi-subunit complexes consisting of different polypeptides. These complexes assemble in precise stoichiometry of its polypeptide partners. To achieve this, the synthesis of different polypeptide complements of a functional protein or a cellular structure is probably tightly controlled to prevent their misexpression. We have investigated whether check-points exist to control the quantity of each polypeptide complement of a multi-subunit functional complex using the thin filament polypeptides of muscle cells

as a model system. In this study we have shown that three- to four-fold overexpression of sTnC mRNA in mouse C2 myotubes did not result in a corresponding increase of the sTnC polypeptide level. The sTnC polypeptide level was increased by only 1.5-fold. We have shown that although the excess sTnC mRNA was efficiently translated, the translation product failed to accumulate in large quantities. A check-point to control sTnC polypeptide level was detected in myotubes. Accumulation of large quantities of unassembled sTnC in the cytoplasm was prevented by preferential rapid degradation of the soluble form of the sTnC polypeptide. The sTnC complexed with the thin filament proteins, in both normal cells and in cells overexpressing sTnC mRNA, were degraded at a rate slower than that of the free sTnC polypeptide. We also investigated whether over-production of sTnC may increase the level of other thin filament polypeptides to maintain the stoichiometry. Results of our studies suggest there was no detectable difference in the cellular level of several thin filament polypeptides like TnT, TnI, α-Tm and α-actin. Therefore, accelerated degradation of any excess unassembled TnC polypeptide appears to be the principle mechanism for maintaining the quantity of sTnC polypeptide so that each polypeptide partner of the thin filament exists in the cell in proper stoichiometry.

In earlier studies we used a different approach for misexpression of sTnC in C2 myotubes [6–8]. The synthesis of sTnC polypeptide was blocked by using an antisense oligodeoxynucleotide. In these studies we also detected that control at the level of polypeptide stability played an important role to maintain the thin filament composition. Short-term inhibition of sTnC synthesis resulted in increased stability of this polypeptide [8]. Therefore, under circumstances of both under-production and over-production of the sTnC polypeptide the check-point works by modulating its rate of degradation. The mechanism to control this step is not known. It is possible that conformational changes in sTnC during its assembly into the thin filament might prevent the formation of the proteasome complex for degradation [25]. It is also not known whether misexpression of other polypeptide partners of the troponin complex will also be regulated by controlling their turn-over rates.

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References

[1] Hastings, K.E.M. and Emerson Jr., C.P. (1982) Proc. Natl. Acad. Sci. USA 79, 1553–1557.
[2] Olson, E.N., Perry, M. and Shulz, R.A. (1995) Dev. Biol. 172, 2–14.
[3] Manhertz, H.G. and Goody, R.S. (1976) Annu. Rev. Biochem. 45, 427–465.
[4] Mathews, M.B., Sonenberg, M. and Hershey, J.W.B. (1996) in: Translational Control (Hershey, J.W.B., Mathews, M.B. and Sonenberg, N., Eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[5] Darnell Jr., J.E. (1982) Nature 297, 365–371.
[6] Thinakaran, G. and Bag, J. (1991) Exp. Cell Res. 192, 227–230.
[7] Ojala, J., Choudhury, M. and Bag, J. (1997) Antisense Nucleic Acid Drug Dev. 7, 31–37.
[8] Choudhury, M. and Bag, J. (1998) Nucleic Acids Res. 26, 4765–4776.
[9] Ojala, J., Choudhury, M. and Bag, J. (1998) Antisense Nucleic Acid Drug Dev. 8, 237–247.
[10] Toulme, J.J. and Helene, C. (1988) Gene 72, 51–55.
[11] Walder, J. (1988) Genes Dev. 2, 502–504.
[12] Nakayama, S. and Kretsinger, R.H. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 473–507.
[13] Parmacek, M.S., Bengur, A.R., Vora, A.J. and Leiden, J.M. (1990) J. Biol. Chem. 265, 15970–15976.
[14] Parmacek, M.S. and Leiden, J.M. (1991) Circulation 84, 991–1003.
[15] Toyota, N. and Shimada, Y. (1983) Cell 33, 297–304.
[16] Berezowsky, C. and Bag, J. (1991) Biochem. Cell Biol. 70, 156–165.
[17] Gahlmann, R., Wade, R., Gunning, P. and Kedes, L. (1988) J. Mol. Biol. 201, 379–391.
[18] Berezowsky, C. and Bag, J. (1992) Biochem. Cell Biol. 70, 691–697.
[19] Schiaffino, S. and Reggiani, C. (1996) Physiol. Rev. 76, 371–423.
[20] Wu, J. and Bag, J. (1998) J. Biol. Chem. 273, 34535–34542.
[21] Thinakaran, G., Ojala, J. and Bag, J. (1993) FEBS Lett. 3, 271–276.
[22] Meinkoth, J. and Wahl, G. (1986) Anal. Biochem. 138, 267–284.
[23] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[24] Laemmli, U. (1970) Nature 227, 680–685.
[25] Wojcik, C. (2001) Trends Cell Biol. 11, 983–990.