Stabilization of slow troponin C polypeptide compensates for its reduced synthesis in antisense oligodeoxynucleotide-treated cells

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ABSTRACT

The expression of genes for contractile proteins during myogenesis is coordinately regulated. Uncoupling the expression of the slow/cardiac troponin C (sTnC) gene from this process with an antisense phosphorothioate oligodeoxynucleotide (ODN) was used to examine the presence of any post-transcriptional mechanisms for regulating muscle protein synthesis. Approximately 70 and 50% decreases in sTnC polypeptide synthesis and mRNA levels, respectively, were achieved after 4 days antisense treatment. This decrease in sTnC polypeptide synthesis was not reflected in a similar decline in the steady-state level of this polypeptide. Extension of the ODN treatment to 7 days was required to produce a substantial decrease in the steady-state level of sTnC polypeptide. Our investigation suggests that during the 4-day treatment, the affected cells stabilized the sTnC polypeptide level by increasing its half-life. However, the stabilizing effect appears to be overridden during prolonged (7 days) antisense ODN treatment. Measurement of the polypeptide synthesis and mRNA levels of several contractile proteins showed no evidence of cross-regulation among the genes to coordinately regulate their expression levels.

INTRODUCTION

Myogenesis is characterized by the expression of an array of muscle-specific genes whose quantitative expression is tightly coupled during the differentiation process. Several studies in the past few years have identified the different myogenic factors involved in muscle cell commitment and differentiation (1,2). However, the post-transcriptional mechanisms governing the stoichiometric expression of contractile proteins are relatively unknown. One approach to examining various levels of control of gene expression during myogenesis is to selectively block the expression of one of the muscle-specific genes. An assessment of how this would influence coordinate expression of the various polypeptides to achieve the desired stoichiometry would help elucidate the presence of various post-transcriptional controls. A simple method to selectively block gene expression is to use an antisense oligodeoxynucleotide (ODN) complementary to unique mRNA sequences of a gene (3–5). This ODN, once internalized (4,6,7), forms an RNA:DNA duplex inside the cell which can either act as a physical block during translation or be subjected to RNase H-mediated degradation, leaving the ODN to function in a catalytic manner. Antisense ODNs have been shown to specifically inhibit gene expression (8–13).

We have studied the expression of the slow/cardiac troponin C (sTnC) gene in the murine C2C12 cell line as a model towards understanding stoichiometric production of the polypeptide components of the myofilament. In C2C12 cells, conversion of myoblasts into myotubes triggers synthesis of sTnC polypeptide. TnC is the smallest subunit of the troponin complex, which is composed of two other troponin proteins, troponin I (TnI) and troponin T (TnT). This complex is involved in Ca2+ regulation during muscle contraction (14). Each of these proteins exists as multiple isoforms whose expression is regulated in a development- and tissue-specific manner (15). Interestingly, all three troponin polypeptides are expressed in a 1:1:1 stoichiometric ratio despite their different regulatory patterns. sTnC and fast TnC (fTnC), the two known isoforms of the TnC polypeptide, are separate gene products. Although sTnC polypeptide is found in both skeletal and cardiac muscle, fTnC occurs exclusively in fast skeletal muscle (16,17).

Studies from this laboratory have demonstrated that the 5′-cap region of TnC mRNA is the best target in achieving 60% inhibition of sTnC synthesis by an unmodified phosphodiester antisense ODN (18) in C2C12 cells. In this study, however, we have employed a more stable phosphorothioate (PS) anti-TnC ODN targeted against the same region of sTnC mRNA. Our studies indicate that anti-TnC ODN treatment results in a decrease in sTnC polypeptide synthesis and its mRNA level. In anti-TnC ODN-treated cells, the half-life of sTnC polypeptide was nearly doubled. Our results, therefore, suggest that compensatory mechanisms may exist in myotubes to overcome the inhibitory effects of the antisense ODN. It is also conceivable that these mechanisms may allow a cell to continue the myogenic program despite attempts to disrupt the process.

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MATERIALS AND METHODS

Cell culture

Mouse skeletal muscle C2C12 myoblasts (ATCC CRL 1772) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL), supplemented with 10% fetal bovine serum (growth medium). Differentiation of myoblasts into myotubes was achieved by addition of DMEM containing 10% horse serum (differentiation medium). Cells were maintained at 37°C for 3–4 days after transfer into the differentiation medium. Cells were maintained under these conditions for different times. Mock-nucleases that may be present in the serum. The cells were treated with DOSPER–ODN complex was added dropwise to the cultures to ensure uniform distribution, without removing 15% SDS–PAGE also showed one major band (results not shown).

ODN treatment of myotubes

Confluent myotube cultures in 12-well dishes were treated with phosphorothioate ODNs (Fig. 1). The ODNs were complexed with DOSPER liposome (Boehringer Mannheim, Indianapolis, IN) according to the instructions of the manufacturer. Briefly, solution A contained 6.5 µg ODN diluted to 50 µl with HEPES-buffered saline, pH 7.2 (20 mM HEPES, 150 mM NaCl). Solution B consisted of 15 µl DOSPER liposome (1 µg/µl) diluted to 50 µl with HEPES-buffered saline. Solutions A and B were combined and mixed gently. They were incubated at room temperature for 15 min to allow complex formation between DOSPER and the ODN.

The differentiation medium was replaced with Optimem, a serum-free medium (Gibco BRL, Grand Island, NY), shortly before transfection. DOSPER–ODN complex was added dropwise to the cultures to ensure uniform distribution, without removing the Optimem medium, such that the concentration of the ODN was 1 µM/ml medium. Following 24 h transfection, Optimem was replaced with the differentiation medium containing 10% minimal nuclease horse serum and 0.5 µM ODN. The horse serum was heat treated at 65°C for 30 min to inactivate any nucleases that may be present in the serum. The cells were maintained under these conditions for different times. Mock-transfected cells were treated with DOSPER without complexing with the ODN and similarly maintained (without ODN) for the desired time of treatment.

Immunoprecipitation

Cellular proteins were labeled as described before and cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS). Samples of cell extracts containing equal amounts of [35S]methionine-labeled polypeptides were precleared with a packed volume of protein A-Sepharose beads (Sigma, St Louis, MO) for 1 h. The supernatant obtained after centrifugation was incubated with monoclonal antibodies to various muscle proteins for 16 h at 4°C (19). Protein–antibody complexes were then further incubated with protein A-Sepharose beads for 2 h. The beads were then washed with RIPA buffer and the bound antigen–antibody complex was eluted at 95°C in SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.2% bromphenol blue, 20% glycerol, 100 mM DTT). Samples were analyzed by 12% SDS–PAGE.

RNA analysis

RNA was isolated from cells using guanidinium thiocyanate (20). The levels of specific mRNAs were measured using reverse transcription-coupled PCR (RT–PCR) reactions. Antisense and sense primers for TnC, TnT and TnI were used to reverse transcribe and subsequently amplify the 5′-region of these mRNAs by PCR. The primers used for reverse transcription for sTnC, fTnC, fTnT and fTnI were d(TGGCGGGCAGTGTTC), d(TTCATCTGGCGGACC), d(CAGTTTCTCTTCGTCA) and d(TGGCGGGCAGTGTTC), respectively. Reverse transcription of 1 µg cellular RNA was performed at 50°C for 1 h with 200 ng of the lower (antisense) primer and 200 U M-MLV reverse transcriptase enzyme (Gibco BRL) in 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 10 mM DTT and 500 µM each dNTPs. The cDNAs were then amplified with 1× reaction buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 mM MgCl2, 100 ng upper (sense) primers, 400 µM dNTPs, 5 U Tsg polymerase (Biobasic). The primers for PCR for sTnC, fTnC, fTnT and fTnI were d(CAGTACGCCTGTCTG), d(TGGCGAGGAACAG), d(AGTGGCCTGCTGCTG) and d(GCTTGAGATCTCAG), respectively. PCR conditions for fTnI were 94°C for 15 s, 50°C for 30 s, 72°C for 30 s for 30 cycles. Touchdown PCR (TD-PCR) was performed for the other troponin mRNAs (21). TD-PCR conditions were the same as above except that the annealing temperatures were consecutively lowered from 55 to 50°C at a rate of 1°C every two cycles of amplification, followed by 18 additional cycles at 50°C. The expected amplified products for TnT (skeletal), fTnI, fTnC and sTnC were 94, 166, 288 and 337 bp long, respectively. The products of RT–PCR were analyzed by 2% agarose gel electrophoresis.

Western blotting

Steady-state levels of sTnC polypeptide were measured using a monoclonal antibody to human cardiac TnC (Research Diagnostics, Flanders, NJ). Protein samples were separated by 12% SDS–PAGE and transferred to a nitrocellulose membrane for 12 h in transfer buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol) at 30 V, 40 mA. The level of sTnC polypeptide was measured by western blotting. Briefly, the antibody–antigen reaction was performed with 1 µg anti-sTnC antibody (Research Diagnostics) per ml phosphate-buffered saline containing 5% skimmed milk.
and 0.1% Tween-20. The sTnC band was visualized with a 1:5000 dilution of alkaline phosphatase-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA).

Measurement of protein stability

Cellular proteins were labeled for 3 h with 100 µCi/ml \[^{35}\text{S}\]methionine (1200 Ci/mmol; ICN) in methionine-free medium. Cells were then maintained in normal differentiation medium supplemented with 20 µM methionine for 0, 2, 4 or 8 h. Cellular proteins were isolated in RIPA buffer at different times following the chase with cold methionine. Samples containing equal c.p.m. were used for immunoprecipitation and analyzed by 12% SDS–PAGE.

RESULTS

Effect of anti-TnC ODN on sTnC polypeptide synthesis

Transfer of myoblasts into differentiation medium induces expression of muscle-specific genes. Expression of one of the major muscle genes in differentiated myotubes, namely the sTnC gene, was targeted for manipulation with an antisense ODN. Cellular proteins were labeled during the last 3 h of the treatment with \[^{35}\text{S}\]methionine and the levels of sTnC polypeptide synthesized during this period were analyzed by immunoprecipitation. Two mismatched antisense (scrambled) sTnC ODNs and another unrelated ODN representing one strand of the MCK enhancer (22) were used to determine the specific action of the anti-TnC ODN. The results indicate that the mock-treated and scrambled and MCK ODN-treated cells did not show any significant decrease in sTnC polypeptide synthesis (Fig. 2 A and F). The antisense ODN, however, conferred an ~70% decrease in sTnC polypeptide synthesis (Fig. 2 A and F). The sTnC band in the antisense ODN-treated samples migrated a little slower than in the lanes with control samples. This may be because the salt concentration in the different immunoprecipitated samples might be slightly different due to residual washing solution. The slower migrating band was identified as sTnC by calculating the mobility of this band. Similar analyses of TnT, TnI, MHC and myogenin polypeptides (Fig. 2 B–E, respectively) revealed that their synthesis was not appreciably altered by the anti-TnC ODN. These immunoprecipitation analyses indicate that the anti-TnC ODN was specific in inhibiting sTnC synthesis.

Alterations in sTnC mRNA levels

The rate of synthesis of a polypeptide usually depends on the cellular mRNA level. Therefore, to determine whether this observed decrease in synthesis of sTnC polypeptide in antisense ODN-treated cells resulted from a decrease in its mRNA levels, RT–PCR was performed using appropriate primers. The levels of several muscle-specific mRNAs, namely fTnC, TnT and TnI, were also measured by RT–PCR. Results of these analyses show that while sTnC mRNA level was reduced by ~50% (Fig. 3 A and E), no reductions in the levels of TnT, fTnC and TnI mRNAs were detected (Fig. 3 B–D, respectively). Furthermore, the control ODNs showed no significant effect on these mRNAs. A dose–response curve with 0.25–2 µg RNA resulted in a reproducible linear response for each primer set (results not shown), thereby indicating that the RT–PCR measurements reflected the levels of individual mRNAs. Additionally, separate dot blots were also performed using the sTnC cDNA clone (16) to confirm these observations (results not shown).

Effect of anti-TnC ODN on the steady-state level of sTnC polypeptide

The steady-state level of a polypeptide inside a cell is maintained by coordination between the rate of synthesis and degradation. To
investigate whether a reduction in the rate of sTnC polypeptide synthesis was also reflected in a decline in its steady-state level, western blotting was performed. The results (Fig. 4A and C) indicate a small decrease in the steady-state level of sTnC polypeptide during the first 4 days of treatment with the anti-TnC ODN. In contrast, there was a much greater decrease in the rate of synthesis of this polypeptide (Fig. 2A). This suggests that although synthesis of sTnC polypeptide was inhibited by ~70%, the steady-state level of this polypeptide was not altered significantly during a 4-day antisense treatment. Maintenance of the steady-state level of this polypeptide suggests the presence of one or more compensatory mechanisms in the cells. However, a dramatic decline in the steady-state level of sTnC was observed following 7 days of anti-TnC ODN treatment (Fig. 4A and C). There was no appreciable decrease in sTnC polypeptide levels in both 4- and 7-day mock-treated and control ODN-treated cells (Fig. 4A–C). These results suggest that although a compensatory process may be functional during the initial stages of the antisense ODN treatment, this mechanism may not be able to sustain the steady-state level of sTnC polypeptide during the longer antisense ODN treatment period.

Stability of sTnC polypeptide

Since the first 4 days of antisense ODN treatment did not significantly reduce the steady-state level of sTnC polypeptide, its stability was analyzed by pulse–chase studies in mock-treated and ODN-treated cells. The levels of [35S]methionine-labeled sTnC polypeptide were measured by immunoprecipitation at different times following the chase with cold methionine. The rates of decay of the sTnC bands were measured to determine the half-life under different conditions. Since sTnC synthesis in anti-TnC-treated cells is reduced to ~30% of that in control cells, to maintain the radioactive signals from these cell extracts within the linear range of autoradiography during the chase, ~70% more of the anti-TnC ODN-treated samples was used for SDS–PAGE in these analyses. Therefore, the intensities of the sTnC bands between mock- and antisense ODN-treated samples during the 0 h chase appears similar. However, this enabled an accurate densitometric comparison of the amount of [35S]methionine-labeled sTnC polypeptide during the various chase times in anti-TnC-treated cells. Densitometric scanning of the sTnC band from an average of three experiments was used to determine the level of labeled sTnC polypeptide after the pulse (0 h) and 2, 4 and
DISCUSSION

In this study, we have shown that liposome-mediated transfer of phosphorothioate antisense ODN into muscle cells was an effective means of blocking target gene expression. The anti-TnC ODN selectively inhibited synthesis of sTnC polypeptide by almost 70%. This reduction in polypeptide synthesis was also accompanied by a ~50% decrease in its mRNA level. These results were reproducible in several (three or more) independent experiments. The concomitant inhibitory effect on both polypeptide synthesis and mRNA level was specific for the target gene. We did not observe any similar inhibition of TnI, Tn and TnT at both the protein and mRNA levels. The lack of inhibition of sTnC polypeptide levels in different control ODN-treated cells further supports the specificity of the anti-TnC ODN.

A balance between the rate of synthesis and degradation of a polypeptide to maintain its steady-state level during myogenesis is probably critical for formation of the myofilament. In these studies, during the 4-day anti-TnC treatment the cells were able to maintain the steady-state level of sTnC polypeptide despite a sharp decrease in the rate of its synthesis. However, extending the antisense treatment to 7 days produced a decline in the steady-state level. We have shown that the myotubes initially compensated for the decrease in the rate of polypeptide synthesis by stabilizing the pool of sTnC polypeptide. This stabilization may allow the myotubes to maintain the stoichiometry of TnC in muscle cells during the early stages of the antisense treatment.

The precise biochemical nature of this checkpoint in sTnC gene expression is not known. Furthermore, additional compensatory mechanisms may exist to regulate muscle protein synthesis in antisense ODN-treated cells. It is, however, interesting to note that the compensatory stabilizing effect on sTnC polypeptide can be overridden by prolonging the antisense treatment to 7 days. This was evident as a decline in the steady-state level of sTnC polypeptide in anti-TnC ODN-treated cells.

Antisense ODNs targeted against a specific gene provide a valuable tool to manipulate expression of the target gene for both basic scientific and therapeutic purposes. The results presented here provide information about uncoupling of the expression of one component of a major multicomponent cellular structure, the sarcomere. We have also shown that the stability of sTnC polypeptide can be regulated in an attempt to sustain a stable level of TnC polypeptide in muscle cells. These data therefore indicate the existence of a hitherto unknown checkpoint in the regulation of troponin C gene expression in muscle cells.
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REFERENCES