Force-expression of Cardiac Troponin I in C2C12 Skeletal Myoblasts Suppresses Myoblast Fusion and Induces Actin Bundle Network Formation

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Abstract: In cardiac muscle of the chicken, slow skeletal form of troponin I (STnI) is initially expressed in the embryo, but it is later replaced by the cardiac form (CTnI). In contrast, in fast and slow skeletal muscles, CTnI is never expressed throughout the course of development. To examine how such a normally absent cardiac isoform behaves in embryonic skeletal muscle if force-expressed, we established several C2C12 skeletal muscle cell lines that stably express CTnI. We used CTnI/9, whose amino acid sequence corresponds to 90% of that of CTnI. In all of these clones, fusion of myoblasts to form myotubes was inhibited. In a clone expressing CTnI/9 at the highest level, networks of thick actin bundles were found. Thus, expression of the TnI isoform, specific for adult cardiac muscle and absent in embryonic skeletal muscle, disturbs normal myogenic differentiation and myofibrillogenesis in skeletal muscle cells.

Key words: Troponin; isoform; DNA transfection; myoblast fusion; actin network.

Introduction. Many of the myofibrillar proteins are organized as isoform families, and during cell differentiation they undergo a multitude of isoform transitions. In embryonic cardiac muscle, slow skeletal muscle troponin I (STnI) is the earliest and the main isoform expressed. As development proceeds, it decreases and cardiac muscle TnI (CTnI) is markedly increased. In embryonic fast and slow skeletal muscles, on the other hand, CTnI is never expressed throughout the course of development. To examine how such unexpressed isoform(s) behaves in embryonic skeletal muscle cells when it is force-expressed, we established several C2C12 skeletal muscle cell lines that stably express CTnI.

Materials and methods. Preparation of CTnI/9 expression plasmid. CTnI/9 cDNA was prepared from a chicken cardiac muscle cDNA library produced in λgt11 as described elsewhere. In comparison with fast TnI (FTnI) and STnI, CTnI contains a longer amino acid extension. Although the CTnI/9 obtained in the present study lacks 23 amino acids of the N-terminal of CTnI, its amino acid sequence corresponds to 90% of that of CTnI, and possesses two actin binding domains in the case of FTnI. Thus, CTnI/9 can be considered to function and behave similarly to CTnI. CTnI/9 cDNA was inserted into EcoRI sites of the expression vector pcDL-SRα296.

Cell Culture and Transfection. C2C12 myoblasts were cultured in DMEM containing 10% fetal calf serum at a concentration of 7 x 10^5 in 8 ml of culture medium in 100-mm dishes. The medium was replaced with Opti-MEM (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) containing 10% fetal bovine serum prior to transfection. To obtain stably expressing CTnI/9 cell lines, a mixture of 3 μg of the CTnI/9 plasmid and 12 μg of pSV2-neo carrying the neo gene for G418 selection was transfected with lipofectamine according to the method of the manufacturer (Life Technologies). At 16 h after transfection, Opti-MEM was substituted with DMEM containing 500 μg/ml of G418. Individual resistant colonies were isolated at 14 d and passaged into stable cell lines. To initiate differentiation, cultures at 80% confluence were transferred to DMEM containing 5% horse serum. Phase contrast microscopy was performed using standard...
Results and discussion. Five G418 resistant differentiation-promoting medium. They were spindle or cells. The results showed that CTnI/9 was synthesized in C2Cl/9a-d thought to be transfected only with pSV2-neo. Thus the resistant and did not synthesize CTnI/9, this clone was respectively, n=5, p>0.01). Since C2Cl/9e cells were G418 resistant and did not synthesize CTnI/9, this clone was transferred to anti-CTnI was detected. In these clones, the expression of the CTnI/9 protein in these clones, total cellular extracts were prepared and analyzed by immunoblotting with anti-CTnI. Avidin-labeled anti-rabbit antibody and biotin-linked horseradish peroxidase (VECTASTAIN Elite ABC kit; Vector Laboratories, Inc., Burlington, CA, U.S.A.) were used to visualize the immunoreactive bands using diamobenzidine and H2O2 as substrates. The images on nitrocellulose sheets were scanned with JX-325M (Sharp Co., Ltd., Osaka, Japan), and the expression level of CTnI/9 in clones was analyzed with a Power Macintosh computer and an ATTO densitograph program (ATTO Corporation, Tokyo, Japan).

Immunoblotting. Immunoblotting was performed according to the standard method. Cells grown on 100 mm dishes were collected with a scraper and suspended in the SDS sample treatment buffer. These samples were subjected to electrophoresis using 11% polyacrylamide gel in the presence of 0.1% SDS according to the procedure of Laemmli. Samples of 20 μl (approximately 10 μg) each were applied. Proteins from the gels were transferred to nitrocellulose sheets and then treated with anti-CTnI. Avidin-labeled anti-rabbit antibody and biotin-linked horseradish peroxidase (VECTASTAIN Elite ABC kit; Vector Laboratories, Inc., Burlington, CA, U.S.A.) were used to visualize the immunoreactive bands using diamobenzidine and H2O2 as substrates. The images on nitrocellulose sheets were scanned with JX-325M (Sharp Co., Ltd., Osaka, Japan), and the expression level of CTnI/9 in clones was analyzed with a Power Macintosh computer and an ATTO densitograph program (ATTO Corporation, Tokyo, Japan).

Results and discussion. Five G418 resistant clones (C2Cl/9a–e) were obtained. To examine the expression of the CTnI/9 protein in these clones, total cellular extracts were prepared and analyzed by immunoblotting with anti-CTnI (Fig. 1). No band was detected in C2Cl12 cells without plasmid transfection, nor in C2Cl/9e cells. However, in C2Cl/9a–d cells, a main band reactive with anti-CTnI was detected. In these clones, the mobilities of the bands of CTnI/9a–d cells were similar to that of CTnI of adult cardiac muscle. Analysis of the expression levels of CTnI/9 revealed that C2Cl/9d produced CTnI/9 at the highest level (expression level of C2Cl/9d [23.9±0.4] was significantly higher than those of C2Cl/9a–c [15.1±0.5, 18.7±0.4 and 19.6±0.5, respectively, n=5, p<0.01]). Since C2Cl/9e cells were G418 resistant and did not synthesize CTnI/9, this clone was thought to be transfected only with pSV2-neo. Thus the results showed that CTnI/9 was synthesized in C2Cl/9a–d cells.

C2Cl/9a–d cells continued to proliferate in the differentiation-promoting medium. They were spindle or flat polygonal in shape. Although in C2Cl12 and C2Cl/9e clones large myotubes were formed within 4 days, in C2Cl/9a–d clones no myotube formation was seen even by 14 days (Fig. 2). These results indicated that myoblast fusion was blocked in cells stably expressing CTnI/9, meaning that the expression of the heterogeneous cardiac muscle protein CTnI in embryonic skeletal muscle cells probably suppressed (directly or indirectly) skeletal myoblast fusion. Similar observations were reported with C2Cl12 cells in which myosin light chain 1 or desmin was disrupted by transfection with antisense DNAs of these proteins. Thus, it appears that such inhibition takes place in a cytoplasmic milieu where the amount of myofibrillar proteins is beyond the physiological range (over-expression or reduction of expression).

To investigate how CTnI/9 is distributed in C2Cl/9a–d cells, 3-day-cultures in the differentiation medium were double stained with anti-CTnI and TMR-phalloidin. Anti-CTnI staining of these cells showed a diffuse pattern, and stress fiber-like structures were reactive to both the antibody and TMR-phalloidin. In contrast, C2Cl12 and C2Cl/9e cells were unreactive to the antibody but did contain TMR-phalloidin positive fibrils (data not shown).

C2Cl/9d cells cultured for 7 weeks were found to possess unusual structures: dotted structures and networks of thick actin bundles (Fig. 3). The latter networks were also reactive with TMR-phalloidin and formed a spherical configuration around the nucleus. Since both of these structures were observed only in C2Cl/9d cells producing CTnI/9 at the highest level, the over-abundance of CTnI/9 can be considered to be closely related to the formation of the CTnI/9 dots and actin networks. In the experiment of tropomyosin-troponin assembly in vitro, it has been shown that purified tropomyosin, one of the constituents of thin filaments, makes mesh-like paracrystals in the absence of troponin, but these paracrystals change their configuration by its addition. Thus, exogenously introduced CTnI/9 may affect the stoichiometry of proteins of thin filaments, and this in turn might have resulted in the construction of actin bundle networks in C2Cl/9d cells.
Our results showed that the accumulation of CTnI/9 suppresses myoblast fusion and myotube formation even in the medium for differentiation. Although multiple TnI isoforms are expressed during embryonic muscles, those never expressed during normal development may interfere with the myogenic differentiation and myofibrillogenesis.

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References