Involvement of Transglutaminase in Myofibril Assembly of Chick Embryonic Myoblasts in Culture

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Abstract. Involvement of transglutaminase in myofibrillogenesis of chick embryonic myoblasts has been investigated in vitro. Both the activity and protein level of transglutaminase initially decreased to a minimal level at the time of burst of myoblast fusion but gradually increased thereafter. The localization of transglutaminase underwent a dramatic change from the whole cytoplasm in a diffuse pattern to the cross-striated sarcomeric A band, being strictly colocalized with the myosin thick filaments. For a brief period prior to the appearance of cross-striation, transglutaminase was localized in nonstriated filamentous structures that coincided with the stress fiber-like structures. When 12-o-tetradecanoyl phorbol acetate was added to muscle cell cultures to induce the sequential disassembly of thin and thick filaments, transglutaminase was strictly colocalized with the myosin thick filaments even in the myosacs, of which most of the thin filaments were disrupted. Moreover, monodansylcadaverine, a competitive inhibitor of transglutaminase, reversibly inhibited the myofibril maturation. In addition, myosin heavy chain behaved as one of the potential intracellular substrates for transglutaminase. The cross-linked myosin complex constituted approximately 5% of the total Triton X-100-insoluble pool of myosin molecules in developing muscle cells, and its level was reduced to below 1% upon treatment with monodansylcadaverine. These results suggest that transglutaminase plays a crucial role in myofibrillogenesis of developing chick skeletal muscle.

Myoblast differentiation is characterized by a well-defined sequence of events leading to the conversion of undifferentiated myoblasts to terminally differentiated myotubes. The spindle-shaped myoblasts initially undergo a phase of proliferation while aligning along the long axes. This is followed by an irreversible withdrawal from the cell cycle and fusion of the cell membrane to form a tube-like syncytium called the myotube, which then matures to become a functional muscle fiber. Concurrently with these cellular changes, muscle-specific myofibrillar proteins are synthesized and subsequently assembled into relatively invariant striated myofibrils (Fischman, 1970; Knudsen and Horwitz, 1977; Wakelam, 1985; Fischman, 1986; Bandman, 1992). Prior to the appearance of nascent myofibrils, nonstriated bundles of microfilaments which are similar to stress fibers in non-muscle cells appear. Sarcomeric configurations of contractile proteins are then organized on these pre-existing bundles of stress fiber-like structures (SFLSs), and this sarcomeric formation proceeds from the periphery toward the center of the myotubes (Peng et al., 1981; Dlugosz et al., 1984; Antin et al., 1986; Wang et al., 1988).

Although the expression of the myofibrillar proteins and their assembly into myofibrils have been extensively studied, little is known about the mechanism controlling the assembly of myofibrillar proteins and the molecular interactions that organize developing myofibrils. The structural considerations and genetic analysis suggest that additional components and reactions are required for the thick and thin filament assembly in vivo (Epstein and Fischman, 1991). Furthermore, it has been reported that myosin-associated proteins of intracellular members of the N-CAM gene family, such as titin, C-protein, 86-kD protein, and myomesin, may function in intra- or inter-filamentous adhesive reactions within the myofibril (Einheber and Fischman, 1990, 1991; Trinick, 1991). However, the possibility has never been suggested that constituents of the myofibril might be stabilized by cross-linking with each other during the formation of myofibrils, although several myofibrillar proteins serve as substrates for cross-linking enzyme like transglutaminase (Cohen et al., 1979; Maccioni and Arechaga, 1986; Huang et al., 1992).

Transglutaminases are calcium-dependent enzymes which catalyze the formation of covalent bonds between protein-bound glutamine residues and primary amino groups,
resulting in protein–protein cross-linking or in amine incorporation into proteins (Folk, 1980; Lorand and Conrad, 1984). The transglutaminase-dependent cross-linking leads to protein polymerization stable and resistant to proteolysis, thereby increasing the resistance of tissues to chemical, enzymatic, and physical degradation. Membrane-bound forms of intracellular transglutaminases have been identified in keratinocytes and their role in cornified envelope formation has been well documented (Rice and Green, 1977; Thatcher and Rice, 1985; Chakravarty and Rice, 1989). Secreted forms of transglutaminases are involved in the clotting of seminal vesicle secretory proteins of the rodents (Williams-Ashman, 1984) and in blood clotting (Laki and Lorand, 1948; Lorand, 1972). In contrast to the clearly defined function of transglutaminase (factor XIII) in blood clotting, the role of tissue transglutaminase, a cytosolic enzyme present in a variety of cells, has not been established yet. Their general function, however, appears to be connected with maintaining the mechanical integrity of tissues (for review see Greenberg et al., 1991).

An interesting observation has been reported that histamine or dapsylcadaverine, a competitive inhibitor of transglutaminase, inhibits the myotube formation. In the treated cultures, myoblast fusion occurred but failed to elongate into normal myotubes resulting in the production of abnormal multinucleated myosacs in vitro. Therefore, it has been suggested that transglutaminase plays an important role in stabilizing the cytoskeletal network of developing myotubes (Bersten et al., 1983). To clarify further the role of tissue transglutaminase in chick myoblast differentiation, especially in myofibrillogenesis, we examined alterations in the activity and cellular localization of transglutaminase as well as its potential substrates.

**Materials and Methods**

**Materials**

Eagle's minimum essential medium (MEM), horse serum, and antibiotics were obtained from Gibco BRL (Gaithersburg, MD). 

- [3H]Putrescine (34.8 Ci/mmol) was obtained from Dupont-New England Nuclear (Wilmington, DE).
- QAE Sepharose from Pharmacia (Piscataway, NJ), enhanced chemiluminescence (ECL) reagent from Amersham Corp. (Arlington Heights, IL), and polyvinylidene difluoride (PVDF) membrane from Millipore Corp. (Bedford, MA). All other reagents including various kinds of antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

The antibody against rabbit skeletal a-actinin, a mouse monoclonal antibody (clone EA-53), reacts specifically with skeletal and cardiac muscle a-actinins. Monoclonal anti-rabbit skeletal myosin antibody (clone MY-32) specific for myosin heavy chain does not stain non-muscle myosin. Monoclonal anti-chick titin (clone T-11) antibody reacts with skeletal and cardiac muscle titins and stains around the region of the A-I junctions.

**Antibody Production**

Transglutaminase was purified from adult chick liver as previously described by Brookhart et al. (1983). To prepare the antibody against transglutaminase, 1 mg of the purified enzyme was electrophoresed on 10% (wt/vol) polyacrylamide slab gels containing SDS under reducing condition (Laemmli, 1970). After briefly staining the gels with Coomassie Blue R-250, the bands corresponding to a molecular mass of about 84 kD were cut out, minced, and injected three times into albino rabbits with 3-wk intervals. Upon immunoblot analysis (Burnette, 1981), the resulting antibody was found to specifically interact with the 84-kD band in the extracts of cultured chick myoblasts, the purified chick liver transglutaminase, and the guinea pig liver transglutaminase (Fig. 1 A). The same antibody, but not the preimmune serum, inhibited the activities of the purified chick liver transglutaminase in a dose-dependent manner (Fig. 1 B), indicating that the anti-transglutaminase antibody prepared in the present study is specific to soluble, tissue-type transglutaminase. For the production of anti-dansyl antibody, keyhole limpet hemocyanin was covalently labeled with dansyl chloride as described (Tijessen and Kurstak, 1979) and injected into albino rabbits as above.

**Myoblast Cultures**

Myoblasts from breast muscles of 12-d chick embryos were prepared as described previously (Chung and Kang, 1990). The cells were plated at a density of 5 × 10⁵ cells/ml in MEM containing 10% (vol/vol) horse serum, 10% (vol/vol) embryo extract, and 1% (vol/vol) antibiotic/antimycotic solution. The culture medium was changed 24 h after plating with the same medium but containing 2% embryo extract. For measurement of myoblast fusion, the cells were fixed with 1% (vol/vol) glutaraldehyde for 30 min, stained with 10% (vol/vol) Giemsa solution for 30 min, and observed under a microscope with a magnification of 250x. Cells were considered fused only if there was clear cytoplasmic continuity and at least three nuclei were present in each myotube. When necessary, cysteine arabinoside was treated to 72–120-h cultures, 12-o-tetradecanoylphorbol acetate (TPA) to 96-h cultures, and monodansylcadaverine (MDC) to 24–80-h cultures at final concentrations of 10 µM, 75 ng/ml and 70-200 µM, respectively. After such treatment, indirect immunofluorescence assays were performed at appropriate time points.

**Transglutaminase Assay**

Myoblasts cultured for various periods were washed with buffer A (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 1 mM EDTA and 1 mM DTT. They were then harvested, sonicated at 4°C for 10 s, and centrifuged for 5 min at 10,000 g. The resulting supernatants, referred to as the cell extracts, were assayed for transglutaminase activity by measuring their ability to incorporate [3H]putrescine into N,N'-dimethylcasein (Placentini et al., 1988). Reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 20 mM DTT, 10 mM CaCl₂, 50 mM NaCl, 70 µM [3H]putrescine (160 Ci/mole) and 1 mg/ml N,N'-dimethylcasein. After incubation at 37°C for 1 h, aliquots of the assay mixtures were spotted onto 3 MM filter papers (Whatman Laboratory Products, Inc., Clifton, NJ). The filter papers were washed three times with 10% (vol/wt) cold TCA and dehydrated with 95% (vol/vol) ethanol. Radioactivity remained on the filter papers was determined using a liquid scintillation counter.

**Immunoochemical Analysis**

Immunoblot analysis was done as described by Burnette (1981) with minor modifications. The cell extracts (100 µg protein) were electrophoresed on 8% slab gels containing SDS under reducing condition, and the proteins in the gels were transferred onto PVDF membranes. The membranes were incubated with 3% (wt/vol) BSA in TBST (100 mM Tris-HCl, pH 8.8, 150 mM NaCl and 0.1% (vol/vol) Triton X-100). They were then reacted with the polyclonal antibody raised against transglutaminase (diluted 1:200 with TBST containing 3% BSA) or with the monoclonal antibody against rabbit skeletal α-actinin or myosin or against chick titin (1:500). After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-IgG (1:5,000). Immunoochemical detection was performed with ECL reagent by following the manufacturer's recommendation.

To immunoprecipitate the MDC-incorporated proteins, 60-h cultures were treated with MDC at 5 µM and incubated for the next 12 h. The cells were then washed three times with buffer A containing 10 mM EGTA and 10 mM putrescine, solubilized by sonication, and centrifuged for 10 min at 10,000 g. The resulting supernatants were incubated with preimmune serum and passed through a protein A-Sepharose column to eliminate non-specific binding. The eluates were centrifuged for 10 min at 10,000 g, and the supernatants were reacted with the anti-dansyl antibody overnight at 4°C. The immune-complexes were precipitated with protein A-Sepharose, washed three times with buffer A containing 0.5% Triton X-100 and 0.6 M NaCl, and solubilized with 2% (wt/vol) SDS and 2% (vol/vol) β-mercaptoethanol. The supernatant samples were then subjected to immunoblot analysis as described above.

To determine whether transglutaminase is indeed involved in cross-linking of any protein in developing muscle cells, 48-h cultures (15 100-mm plates seeded at a concentration of 10³ cells/ml for each preparation) were incubated for 24 h in the presence and absence of 100 µM MDC. After incubation, the cells were washed three times with buffer A containing...
10 mM EGTA and 10 mM putrescine and then with 10 mM sodium phosphate buffer (pH 7.5) containing 1% Triton X-100, 150 mM NaCl, and 0.1 mM PMSF. The resulting samples were disrupted in a Dounce homogenizer and centrifuged at 15,000 g for 10 min. The pellet was washed with 10 mM sodium phosphate buffer (pH 7.2) containing 0.6 M NaCl, 5 mM MgCl₂, and 0.1 mM PMSF, solubilized by homogenization in 2% SDS and 2% β-mercaptoethanol, and subjected to electrophoresis on 3% slab gels containing SDS. The gel pieces containing the high molecular weight proteins (i.e., the proteins that stacked just beyond the gel wells) were cut out, minced and digested with 50 μg/ml of trypsin for 10 min at 37°C. The digested products were subjected to electrophoresis on 8% slab gel containing SDS and then to immunoblot analysis using anti-myosin antibody.

**Immunofluorescence Microscopy**

Myoblasts grown on collagen (5 mg/ml)-coated coverslips were rinsed twice with PBS, fixed with 3.7% (wt/vol) paraformaldehyde for 10 min, and washed with 0.15 M glycine in PBS. For detection of titin, the cells were fixed with 95% ethanol. They were then permeabilized with 0.5% Triton X-100 in PBS for 5 min and blocked with 3% BSA in PBS. The resulting cells were incubated with appropriate combinations of the antibodies that had been diluted as above with PBS containing 1% BSA and washed several times with PBS. They were then stained with FITC- and/or TRITC-conjugated anti-IgGs (1:200). The samples were mounted with glycerol containing 10 mM o-phenylenediamine and observed under a fluorescence microscope (Olympus).

**Ligand Blot Analysis**

Myoblasts cultured for 60 h were disrupted by sonication, electrophoresed, and transferred onto PVDF membranes as described above. The membranes were incubated with 3% BSA in buffer A and then with 10 μg/ml of the purified chick liver transglutaminase in buffer A containing 10 mM CaCl₂ and 2 mM DTT. After washing with buffer A containing 10 mM CaCl₂ and 0.5% Triton X-100, the membranes were incubated with anti-transglutaminase antibody. Protein substrates interacted with transglutaminase were then visualized by treatment of ECL reagents.

**Quantification of Cross-linked Myosin**

Myoblasts cultured for 60 h were incubated for 12 h in the presence and absence of 70 μM MDC. After incubation, the cells were suspended in PBS containing 0.5% Triton X-100, homogenized in a Dounce homogenizer, and centrifuged for 10 min at 15,000 g. The resulting Triton-insoluble pellet was solubilized by boiling for 5 min in PBS containing 2% SDS and 2% β-mercaptoethanol.

To separate the cross-linked, high molecular weight form of myosin from monomeric myosin molecules, the boiled sample (0.8 mg) was subjected to gel filtration on a Sephacryl S-300 column (0.75 × 40 cm) that had been equilibrated with PBS containing 2% SDS and 2% β-mercaptoethanol. Fractions of 0.8 ml were collected at a flow rate of 5 ml/h. Aliquots of the fractions (30 μl each) were subjected to electrophoresis on 10% polyacrylamide slab gel containing SDS under reducing condition followed by immunoblot analysis using anti-myosin antibody.

Proteins, that had been eluted in the void volume and therefore should have much larger sizes than monomeric myosin and could not be transferred onto nitrocellulose membranes, were pooled and referred to as fraction-1. The fractions containing immune-reactive, monomeric myosin molecules were also pooled and referred to as fraction-2. Each of the fractions was then spotted on nitrocellulose papers using a dot-blot apparatus (Schleicher & Schuell, Inc., Keene, NH). After the sampling, the papers were washed sequentially with 1% glutaraldehyde, 1 M glycine, and PBS. They were then subjected to immunoreaction with anti-myosin antibody. Amounts of myosin in the fractions were then quantified by scanning the sampling dots using a densitometer (model GS300; Hoefer Scientific Instruments, San Francisco, CA).

**Results**

**Changes in the Transglutaminase Activity during Myogenesis**

Transglutaminase in cultured myoblasts has been suggested to play an important role in the formation of the normal elongated myotubes following the cell fusion (Berenst et al., 1983). In an attempt to clarify the role of transglutaminase in myofibrillogenesis, we first examined whether the enzyme activity changes during the course of the myogenic process. Extracts were prepared from myoblasts that had been cultured for various periods and assayed for their ability to incorporate [3H]putrescine into N,N′-dimethylecine. As shown in Fig. 2A, the enzyme activity initially declined, particularly at the time of burst of myoblast fusion, but gradually increased thereafter. We then examined whether the changes in the enzyme activity are due to alterations in the expression of transglutaminase in differentiating myoblasts or to other unknown reason(s). Fig. 2B (panel c) shows that the protein level of the enzyme also decreases at the time of initiation of fusion and then increases gradually, indicating that the changes in the enzyme activity are due to the fall-and-rise of the expression of transglutaminase during the culture periods. As a control, the changes in the protein levels of myosin (panel a) and α-actinin (panel b) during the myogenic period are also shown.

Similar changes in the activity of transglutaminase were detected in the extracts of breast muscle, which was obtained from 8–20-d chick embryos. The enzyme activity initially declined to a minimal level in the extract from 14-d embryos and then increased to become maximal in 18–20-d embryos (data not shown). Furthermore, the changes in the enzyme activity occurred almost in parallel with the alterations in its protein level in the muscle extracts as determined by immunoblot analysis using the anti-transglutaminase antibody (data not shown). Since most myoblasts in skeletal muscle of 14-d embryos are known to start rapid fusion (Hermann et al., 1970), the changes in the transglutaminase activities in both the cultured cells and the embryonic muscle tissue appear to correlate with the myogenic differentiation.

**Cellular Localization of Transglutaminase during Myogenesis**

To determine the cellular localization of transglutaminase during the assembly of myofibrils, myoblasts were cultured for various periods and subjected to indirect immunofluorescence microscopy. The distribution of transglutaminase was dramatically changed from the diffuse pattern to the definitively striated sarcomeric banding pattern. In 24-h cultures, mononucleated myoblasts, which were still skeletal myosin-negative, exhibited diffuse fluorescence throughout the cytoplasm (Fig. 3A, inset). Between 48 and 72 h of the cultures, the enzyme appeared for a brief period as longitudinal arrays in immature multinucleated myotubes as well as in moderately elongated mononucleated myoblasts. The longitudinal arrays of transglutaminase at this stage (Fig. 3C) appeared coincident with the periodically interrupted arrays of SFLSs stained with the anti-α-actinin antibody (Fig. 3D). As the striated myofibrils increased within the myotubes, distinct banding pattern of transglutaminase as broad doublets emerged along the longitudinal filaments, which became increasingly segregated into bundles between myofibrils (Fig. 3E). In addition, it could also occasionally be seen in a fully elongated postmitotic mononucleated myoblast (Fig. 3B).
Figure 1. Specificity of the polyclonal antibody raised against the purified chick liver transglutaminase. (A) Guinea pig liver transglutaminase (lane a), the purified chick liver transglutaminase (lane b) and the extract from 72-h cultures of chick myoblasts (lane c) were subjected to immunoblot analysis using the antisera prepared against the purified chick liver transglutaminase. The size markers used were rabbit muscle myosin (205 kD), Escherichia coli β-galactosidase (116 kD), rabbit muscle phosphorylase b (97 kD), and egg albumin (45 kD). (B) The activity of the purified chick liver transglutaminase was assayed in the presence of increasing amounts of the antiserum (○) or preimmune serum (△).

To determine more precisely the localization of transglutaminase doublet in sarcomeres, the cultured cells were also stained with anti-transglutaminase antibody (Fig. 4, A and C) and together with the antibody against α-actinin (Fig. 4 B) or myosin (Fig. 4 D). The transglutaminase bands were localized within the A bands as doublets flanked by the M lines. Furthermore, they exactly coincided with the myosin doublets (Fig. 4, C and D).

We then investigated the temporal sequence of the appearance of transglutaminase cross-striations by double immunofluorescence assays using the antibodies against transglutaminase and titin, which is known as one of the earliest markers for sarcomere formation (Hill et al., 1986; Tokuyasu and Maher, 1987; Colley et al., 1990). In the early mononucleated myoblasts (i.e., 40-h cultures), transglutaminase was more intensely stained than titin by their respective antibodies (Fig. 5, A and B, arrowheads). In the elongated myoblasts and early myotubes, both transglutaminase and titin appeared with about the same intensity as interrupted filamentous structures that resemble SFLSs longitudinally aligned along the cell edges (Fig. 5, A and B, arrows). As the cells further developed (i.e., 60-h cultures), a periodic, perpendicular disruption in the filamental structures became evident and the structures that resemble newly emerging primitive sarcomeres were elicited (Fig. 5, C and D). The thin bands on tandem arrays, stained with the anti-transglutaminase antibody, appeared to have almost constant lengths like those seen in fully matured myofibrils. The anti-titin antibody showed more discrete, developing patterns of sarcomere, but segregation of titin into doublets was not apparent yet. As the cells matured (i.e., 80-h cultures), however, both transglutaminase and titin appeared as typical sarcomeric striations, of which transglutaminase is in the A bands and titin is in the A-I.
Colocalization of transglutaminase and myosin in developing myofibrils. 96-h cultures were fixed and incubated with the anti-transglutaminase antibody (A and C) plus anti-α-actinin antibody (B) or anti-myosin antibody (D). Transglutaminase was then visualized by treatment of FITC-conjugated goat anti-rabbit IgG, and α-actinin and myosin were by TRITC-conjugated donkey anti-mouse IgG. B and D are the double stained images of A and C, respectively. Arrowheads indicate the Z lines, and arrows show the M lines. Note the strict colocalization of transglutaminase and myosin in every sarcomere. Bars, 5 μm.

junctions (Fig. 5, E and F). Thus, a temporal and topographical correlation in the sarcomeric cross-striation appears to exist in the distribution of titin and transglutaminase in elongated mononucleated myoblasts and multinucleated myotubes.

Association of Transglutaminase with Myosin Thick Filaments

The colocalization of transglutaminase and myosin as revealed by immunofluorescence assay suggests that the enzyme is in association with myosin thick filaments in sarcomeres. Moreover, the distance between the adjacent transglutaminase bands appears to vary depending on the degree of contraction-relaxation of myofibrils, while the width of each transglutaminase doublet remains constant (see Fig. 3 F, arrows). To clarify further the association of transglutaminase and myosin thick filaments, TPA was added to the cultures and its effect on localization of transglutaminase was examined again by indirect immunofluorescence microscopy. It has well been documented that TPA induces the sequential disassembly of myofibrils, in which et-actin thin filaments are disrupted first and myosin thick filaments later (Lin et al., 1987, 1989). The 96–120-h cultures that had not been exposed to TPA showed a typical sarcomeric pattern when stained with the antibodies against myosin, α-actinin, titin, and transglutaminase (see above). By 5 h after TPA treatment to 96-h cultures, α-actinin began to form the cortical α-actinin containing bodies (CABs) (Fig. 6 B) which consist of α-actin and sarcomeric α-actinin core rimmed with vinculin and talin (Lin et al., 1987). Thus, it appears that the I–Z–I complexes have been disrupted from sarcomeres, in accord with the earlier demonstrations by Lin et al. (1987). Although the actin thin filaments were disrupted, the staining with the anti-myosin antibody elicited normal tandem A bands (Fig. 6 H). As expected, the transglutaminase doublets also remained intact and longitudinally aligned (Fig. 6, A and G). By 12 h, CABs were almost completely disappeared (Fig. 6 D), indicating that elimination of I–Z–I complexes, sarcomeric α-actin and α-actinin, has been completed (Lin et al., 1987). During the same period, however, most of both transglutaminase and myosin in tandem A bands were not much perturbed and maintained their linear alignment (Fig. 6, I and J). By 20 h, morphology of the cells completely changed into closely packed multinucleated myostas and CABs as well as sarcomeric α-actinin structures disappeared (Fig. 6, E and F). Both myosin and transglutaminase relocalized into amorphous patches instead of tandem A bands (Fig. 6, K and L). Some myostas exhibited dispersed thick filaments along the edges of the amorphous patches with transglutaminase and myosin being strictly colocalized. These observations clearly suggest that transglutaminase is associated with myosin thick filaments.

Effects of Transglutaminase Inhibitors on Myofibril Assembly

To investigate the involvement of transglutaminase in myofibril assembly, 24-h cultures were treated with MDC, a
Effects of TPA on the localization of transglutaminase, α-actinin and myosin in myofibrils. TPA was added to 96-h cultures at a final concentration of 75 ng/ml. After the treatment, the cells were further cultured for the next 5 h (A, B, G, and H), 12 h (C, D, I, and J) and 20 h (E, F, K, and L). They were then fixed and incubated with antibodies against transglutaminase (A, C, E, G, I, and K), α-actinin (B, D, and F) and myosin (H, J, and L).

Transglutaminase was then visualized by treatment of FITC-conjugated goat anti-rabbit IgG, and α-actinin and myosin were by TRITC-conjugated donkey anti-mouse IgG. A, C, E, G, I, and K are the double stained images of B, D, F, H, J, and L, respectively. Arrows (B) indicate cortical α-actinin containing bodies (CABs). Note that both the transglutaminase and myosin bands still remain intact while the Z lines are disrupted in the cells treated with TPA for 12 h. Bars, 10 μm.

Figure 6.

Competitive inhibitor of transglutaminase, at a final concentration of 200 μM and further cultured for the next 48 h. In accord with the observation by Bersten et al. (1983), the MDC-treated cells formed compact multinucleated myosacs with little cytoplasm. Upon immunofluorescence assays using the antibodies against transglutaminase, myosin, α-actinin, and titin, these myosacs showed no discernible staining pattern but some condensed fluorescence in their cytoplasm (data not shown).

When 60-h cultures were treated with MDC at 70 μM, most of the fully elongated myoblasts and immature myotubes were turned into abnormal multinucleated myosacs within 12 h after the treatment. The myosac was devoid of myofibrils but displayed SFLSs as shown by staining with antibody against α-actinin (Fig. 7 B). Furthermore, arrays of the SFLSs coincided with the longitudinally oriented fine filamentous structures stained with the anti-transglutaminase antibody (Fig. 7 A). Myosin and titin were colocalized with transglutaminase in non-striated thin fibrillar structures, which largely ran parallel to the long axes of the myosacs (Fig. 7, C–F). These effects of MDC could be reversed upon removal of the agent (Fig. 8, A and B) and the normal sarcomeric pattern for each constituent became again evident within the following 24 h (Fig. 8, B, inset).

However, when MDC was treated to the fully matured myotubes (i.e., 80-h cultures), it showed little or no effect on the myofibrillar structure (Fig. 8, C and D). These results clearly suggest that MDC interferes with the myofibril assembly by blocking the activity of transglutaminase and consequently arrests myotubes in the premyofibril stage. Thus, transglutaminase may play an important role in myofibrillogenesis, possibly by cross-linking certain myofibrillar proteins.

Identification of Protein Substrates for Transglutaminase

In order to identify potential substrates for transglutaminase, ligand blot analysis was performed as described under Materials and Methods. Proteins in the extracts of 60-h cultures were separated by polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol, transferred onto PVDF membranes, and incubated with and without the purified transglutaminase and then with...
the anti-transglutaminase antibody. As shown in Fig. 9 A, a major band of about 200 kD strongly interacted with the antibody. An additional major band of 84 kD was detected in the same membranes, but this turned out to be an endogenous transglutaminase in the extract, since it could also be detected without the treatment of the purified enzyme. A few minor bands were also detected but without consistency. However, all the protein bands except the endogenous transglutaminase band were abolished when EGTA and putrescine were simultaneously treated with the purified transglutaminase to the membranes. These results clearly suggest that transglutaminase interacts predominantly with the 200-kD polypeptide perhaps by exerting its enzymatic activity.

It has been reported that the rod portion of myosin molecules can serve as a substrate for microbial transglutaminase (Huang et al., 1992). In addition, the present demonstrations that localization of transglutaminase in sarcomeres exactly coincides with that of myosin suggest that transglutaminase may use myosin as its major substrate. Therefore, it appears possible that the 200-kD polypeptide identified by ligand blot analysis is the myosin heavy chain. To test this possibility, extracts from the 60-h cultures that had been exposed to 5 \( \mu M \) MDC for 12 h were subjected to immunoprecipitation using the anti-dansyl antibody. MDC-incorporated proteins were then electrophoresed in the presence of SDS and \( \beta \)-mercaptoethanol, transferred onto PVDF membranes, and incubated with the antibodies against various myofibrillar proteins, such as myosin, \( \alpha \)-actinin, and titin. As shown in Fig. 9 B, the 200-kD protein that must have been dansylated and hence precipitated by the anti-dansyl antibody strongly reacted with anti-myosin antibody but not with others. These results indicate that the 200-kD protein is the myosin heavy chain.

To determine whether myosin molecules can indeed be cross-linked by intracellular transglutaminase, 48-h cultures were incubated for 24 h in the presence and absence of MDC and subjected to analysis for the appearance of trypsin-sensitive, high molecular weight protein complexes that could be stained with anti-myosin antibody. Since the protein complexes before trypsinization stacked just beyond the wells of a 3% polyacrylamide gel containing SDS and \( \beta \)-mercaptoethanol (data not shown), their sizes appeared to exceed 2,000 kD. Fig. 9 C shows that amounts of the trypsinated, high molecular weight complexes interacting with anti-myosin antibody are greatly reduced in the MDC-treated cells, in which the activity of transglutaminase must have been inhibited. Furthermore, the total amount of the proteins obtained before trypsinization from the cells treated with MDC was also significantly reduced (i.e., by about 40%) as compared with that from the untreated cells, despite the fact that the MDC treatment showed little or no effect on the total number of nuclei in the cells when counted 24 h after the treatment (data not shown).
These results indicate that myosin heavy chains are used as a substrate for transglutaminase in developing muscle cells, although it remains unclear whether the protein molecules are cross-linked with each other or with other myofibrillar proteins.

**Quantification of Myosin Molecules Cross-linked by Transglutaminase**

To quantify the myosin molecules that had been cross-linked by transglutaminase during myofibrillogenesis, the Triton-insoluble proteins of the cultured myoblasts were solubilized by boiling in SDS and β-mercaptoethanol and separated into two fractions using a Sephacryl S-300 column: fraction-1 recovered from the void volume and fraction-2 containing monomeric myosin molecules. Whether or not the cells were treated with MDC, we could not detect any monomeric form of myosin in the fraction-1 upon immunoblot analysis using anti-myosin antibody (Fig. 10 A, lanes 1 in panels b and c). However, when the same fraction-1s containing high molecular weight proteins were spotted on nitrocellulose papers, they showed immunoreaction with the antibody in a dose-dependent manner (Fig. 10 B, lanes 1 in panels b and c). Furthermore, the fraction-1 obtained from MDC-treated cells contained significantly lower amount of the immunoreactive materials than that from control cells. As a control, the purified myosin molecules were also subjected to boiling in SDS, gel filtration, and immunoblot and dot-blot analyses as described above. As shown in Fig. 10 (lanes 1 in panel a of A and B), there was no self-aggregation of monomeric myosin molecules to generate the fraction-1-like proteins through the entire experimental procedure.

The percentage of myosin in the fraction-1 (i.e., cross-linked myosin) out of total amount of the Triton-insoluble myosin molecules was then estimated by scanning the spots in Fig. 10 B using a densitometer. Upon three independent experiments, the cross-linked myosin in non-treated cells was calculated to be 4.9% of the total myosin molecules while that in MDC-treated cells was reduced to 0.89%. These results suggest that MDC blocks the myofibril assembly perhaps by inhibiting the activity of transglutaminase and hence by interfering with the formation of the small cross-linked fraction of myosin in developing muscle cells.

**Discussion**

In smooth, cardiac, and skeletal muscle tissues of guinea pig, a protein with acyl transfer activity has been identified and shown to be antigenically similar to the transglutaminase isolated from liver (Chung, 1972). Also the presence of transglutaminase activity in chick embryonic myoblasts has been demonstrated (Bersten et al., 1983). We report here that both the activity and protein level of transglutaminase in cultured chick myoblasts change during the course of myogenic differentiation. Noteworthy is the finding that the enzyme activity falls to a minimal level at the time of the onset of myoblast fusion and then increases gradually. Although there is no direct evidence, the initial decrease in the transglutaminase activity is interesting when considered in light of the earlier observations. For example, the proliferating myoblasts have a well-developed and highly interconnected internal filament networks. When the cells leave the proliferation cycle and prepare for fusion, cytoskeletal networks are extensively reorganized and destabilized (Fulton et al., 1981; Shimada and Isobe, 1986). The decreased transglutaminase activity may account for this reorganization and destabilization of cytoskeletal network. However, the activity of transglutaminase does not seem to be directly related with the fusion itself, since myoblasts treated with the inhibitors of the enzyme are capable of fusion although the morphology of the cells change into abnormal multinucleated myotubes (Bersten et al., 1983).

When the replicating myoblasts leave the proliferating stage, they are rapidly elongated, coordinately synthesize myofibrillar proteins, and start to assemble long, fine myofibrils, irrespective of fusion (Chi et al., 1975a, b; Hill et al., 1986; Lin et al., 1994). In this early stage of myofibril assembly, many myofibrillar proteins, such as α-actinin (Jokusch and Jokusch, 1980; Endo and Masaki, 1984) and tropomyosin (Holtzer et al., 1972), are detected in association first with nonstriated and subsequently with striated myofibrils. Of interest is the finding that transglutaminase is also distributed in the nonstriated filamental structures prior to its appearance in cross-striations. These thin, longitudinally arrayed filamental structures stained with the anti-transglutaminase antibody are likely the FSLs, which

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*Figure 10.* Separation of cross-linked myosin from monomeric myosin molecules. The Triton-insoluble proteins were obtained from 60-h cultures that had been incubated in the absence (panels b) and presence (panels c) of 70 μM MDC for the next 12 h. The resulting proteins were solubilized by boiling in PBS containing 2% SDS and 2% β-mercaptoethanol and chromatographed on a Sephacryl S-300 column equilibrated with the same buffer. (A) The fraction-1 recovered in the void volume (numbers 1) and the fraction-2 containing monomeric myosin (numbers 2) were subjected to electrophoresis on 6% polyacrylamide slab gels containing SDS and β-mercaptoethanol followed by immunoblot analysis using anti-myosin antibody. (B) Increasing amounts of the same fractions (i.e., 5, 20, 80, and 320 μl from top to bottom) were spotted on nitrocellulose papers using a dot-blot apparatus and incubated with the antibody and then with horseradish peroxidase-conjugated anti-IgG. The purified myosin was treated as above as a control (panels a).
are characterized by a punctate pattern upon staining with the anti-α-actinin antibody. It is widely accepted that SFLS provides the initiation site for myofibril formation (Dlugosz et al., 1984; Antin et al., 1986; Wang et al., 1988; Handel et al., 1989; van der Ven et al., 1993). Therefore, it is possible that transglutaminase may play an important role from the early stage of myofibril formation.

Shortly after the appearance of nonstriated filamentous structures, transglutaminase was detected in definitively sized sarcomeres in association with the myosin thick filaments. Several lines of evidence support the conclusion that transglutaminase and myosin are associated with each other in sarcomeres: (a) the indirect immunofluorescence assay revealed that transglutaminase was localized in the sarcomeric A band as a doublet flanked by the M line, and the double staining revealed that transglutaminase and myosin were strictly colocalized in the A band; (b) irrespective of whether myofibrils contract or relax, the width of transglutaminase bands appeared constant, while the distance between the doublets varied depending on the degree of contraction; (c) when the myofibrils were perturbed with TPA, which induces selective and sequential disassembly of thin and thick filaments, transglutaminase showed a nearly identical behavior to myosin thick filaments.

In a previous report, Huang et al. (1992) have demonstrated that skeletal myosin can serve as a substrate for transglutaminase. The rod portion of myosin filaments from rabbit skeletal muscle was quickly cross-linked with each other by the action of microbial transglutaminase and hence the cross-linked myosin filaments could no longer be extracted by high salt concentrations. In addition, human plasma transglutaminase (fibrinoligase or factor XIIIa) has been shown to be capable of cross-linking rabbit skeletal myosin filaments to form high molecular weight covalent polymers of myosin (Cohen et al., 1979). Using ligand blot analysis, we also found that the purified chick liver transglutaminase interacts most specifically with the 200-kD myosin heavy chain but not in the presence of EGTA or putrescine. Furthermore, the 200-kD protein molecules in the developing myofibrils were found to be exclusively labeled with MDC, which is widely used as a fluorescent tracer for the glutamyl substrates of transglutaminase (Selkoe et al., 1982; Tokunaga et al., 1993). Therefore, we suggest that myosin heavy chain is one of the potential in vivo substrates of chick myoblast transglutaminase.

When myofibrils are isolated from muscle, most of the proteins can be solubilized by SDS under reducing conditions (Ettlinger et al., 1976). However, there has been little attention to how much of the myosin in myofibrils is cross-linked by transglutaminase and hence can not be solubilized by SDS. In the present studies, we demonstrate that the cross-linked myosin constitutes approximately 5% of the total Triton X-100-insoluble myosin molecules. Of particular interest is the finding that treatment of MDC to cultured myoblasts prevents the formation of the cross-linked myosin. Since MDC inhibits the assembly of myofibrils in developing muscle cells but not interferes with the structure of mature myofibrils (i.e., 60- versus 80-h cultures; see Fig. 8, A and D), this small cross-linked fraction of myosin may be critical in the process of myofibril assembly, such as in maintaining the stability of the A band.

In this regard, an important, unanswered question is whether myosin molecules are cross-linked with each other or with other myofibrillar proteins. If in case the reaction site of transglutaminase is limited only to myosin molecules, it seems hard to expect that a low level of random cross-linking of myosin rods plays a critical role in myofibril assembly. Therefore, it is tempting to speculate that the reaction site may be in specific area of the A band, such as the zone of interconnection in myosin thick filaments covering the cross-bridge bearing region (Squire and Harford, 1982; Magid et al., 1984; Suzuki and Pollack, 1986). There have been a number of implications that the inner two thirds of the zone of interconnection coincides with a set of transverse strips equally spaced at 43 nm, harboring the A band-associated proteins, such as C-proteins, H-proteins, and X-proteins (Squire and Harford, 1982; Dennis et al., 1984; Bähler et al., 1985; Suzuki and Pollack, 1986). In addition, it has been reported that titin has the substructure with a 43 nm periodicity in the A band (Fürst et al., 1989) and interacts with the A band-associated proteins as well as the myosin filaments in vitro (Fürst et al., 1992; Koretz et al., 1993; Soteriou et al., 1993). Thus, it seems possible that transglutaminase may be involved in cross-linking of myosin molecules to the A band-associated proteins and/or titin. To address the question whether these proteins are in situ substrates for transglutaminase, detailed studies on ultrastructural localization of the enzyme as well as direct biochemical analysis are required.

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