Coordinate Expression of α-Tropomyosin and Caldesmon Isoforms in Association with Phenotypic Modulation of Smooth Muscle Cells*

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 Isoforn diversity of tropomyosin is generated from the limited genes by a combination of differential transcription and alternative splicing. In the case of the α-tropomyosin (α-TM) gene, exon 2a rather than exon 2b is specifically spliced in α-TM-SM mRNA, which is one of the major tropomyosin isoforms in smooth muscle cells. Here we demonstrate that expressions of α-tropomyosin and caldesmon isoforms are coordinately regulated in association with phenotypic modulation of smooth muscle cells. Molecular cloning and Western and Northern blotting have revealed that in addition to the down-regulation of β-TM-SM, α-TM-SM converted to α-TM-F1 and α-TM-F2 by a selectional change from exon 2a to exon 2b during dedifferentiation of smooth muscle cells in culture. Simultaneously, a change of caldesmon isoforms from high Mᵣ type to low Mᵣ type was also observed by alternative selection between exons 3b and 4 in the caldesmon gene during this process. In contrast, cultured smooth muscle cells maintaining a differentiated phenotype continued to express α-TM-SM, β-TM-SM, and high Mᵣ caldesmon. In situ hybridization revealed specific coexpression of α-TM-SM and high Mᵣ caldesmon in smooth muscle in developing embryos. These results suggest a common splicing mechanism for phenotype-dependent expression of tropomyosin and caldesmon isoforms in both visceral and vascular smooth muscle cells.

It is important to elucidate the molecular mechanism of phenotypic modulation of smooth muscle cells (SMCs)1 such as vasculogenesis, enterogenesis, atherosclerosis, hypertension, and leiomyogenic tumorigenesis. The SMCs are derived from mesodermal precursors, but the intracellular and extracellular factors determining the SMC lineage and its phenotype remain unclear. The search for molecular parameters indicating SMC phenotype is a first step in analyzing phenotypic modulation of SMCs. Several cytoskeletal and contractile proteins are such candidates. Among them, changes of actin (1, 2), caldesmon (CaD) (1, 3, 4), myosin heavy chain (5, 6), and vinculin/meta-vinculin (1, 7) isoforms are closely associated with phenotypic modulation of SMCs. Recent studies have focused on the gene regulation of such parameters (8–14). In addition to these isoform changes, expression of α-smooth muscle actin (α-SM actin) (15, 16), CaD (1, 3, 4), myosin heavy and light chains (5, 6, 17), meta-vinculin (1, 7), SM22, and calponin (18–20) have been reported to be up-regulated during differentiation of SMCs, but down-regulated during dedifferentiation, suggesting the involvement of SMC phenotype-dependent transcriptional regulation in the SMC-specific parameter genes. In fact, the transcriptional machineries of α-SM actin (8, 9), CaD (12), myosin heavy chain (13), SM22 (21), and calponin (22) have been partially characterized.

Tropomyosin (TM) is a predominant helical protein that binds to actin groves. Recent evidence suggests that Ca²⁺-dependent actin-myosin interaction in smooth and nonmuscle cells is controlled by myosin- and actin-linked dual regulation. In this regulation, the TM and CaD are involved as actin-linked regulators (23–25). Also, it has been proposed that both proteins are essential for the reorganization of the actin cytoskeleton mediated by stabilization of microfilaments (26–28). Therefore, TM, in addition to CaD, plays a vital role in motile events. One- and two-dimensional gel electrophoreses revealed multiple TM isoforms associated with morphological changes and tumorigenic transformation (29–31). cDNA and genomic DNA analyses have also shown a diversity of TM mRNAs generated from the limited TM genes by a combination of differential promoter usage and alternative splicing (reviewed in Ref. 32). Of these, some of the TM isoforms accumulate in a tissue-specific manner. It has been demonstrated that the chicken and rat β-TM genes generate multiple transcripts. A pair of internal exons (exons 6a and 6b) are spliced in a mutually exclusive manner, and their utilization is regulated during differentiation of skeletal muscle cells; exons 6a and 6b are used in myoblasts and myotubes, respectively (33, 34). In the case of the α-TM genes, exon 2b rather than exon 2a (termed exons 3 and 2, respectively, in Ref. 35) is spliced in the high Mᵣ α-TM mRNAs in all cell types except for SMCs (35, 36); exon 2a is specifically selected in SMCs. The selection of exon 2a is therefore considered to be an SMC-specific event. The splicing mechanism of the α-TM genes has been also studied (37, 38); however, there are no reports regarding isoform interconversion of TM in association with phenotypic modulation of SMCs.

Here we have been the first to demonstrate expressive...
change of TM isoforms depending upon SMC phenotype. During
dedifferentiation of SMCs in primary culture, the SMC-type
α-TM (α-TM-SM) converted to fibroblast-type 1 and 2 α-TM isoforms (α-TM-F1 and α-TM-F2) by a selectional change from exon 2α to exon 2b, while expression of SMC-type β-TM (β-TM-SM) was down-regulated. Under culture conditions in which differentiated SMCs were caused to dedifferentiate by serum or platelet-derived growth factor-BB (PDGF-BB), expression change of CaD from high M, (h-CaD) to low M, form (I-CaD) was coincident with that of TM. In contrast, expression of α-TM-SM and h-CaD was maintained in differentiated SMCs cultured on laminin-coated dishes without serum. In situ hybridization revealed coexpression of α-TM-SM and h-CaD mRNAs in smooth muscle in developing embryos. The present results, therefore, suggest that the α-TM and CaD genes are coordinately regulated in a SMC phenotype-dependent manner. We further discuss the molecular mechanism of such gene expressions including transcription and splicing during phenotypic modulation of SMCs.

**MATERIALS AND METHODS**

**Cell Culture**—We prepared SMCs from 15-day-old chick embryo giz-
brids. Briefly, gizzard muscles were carefully separated from serosa and tunica mucosa, minced with scissors, and incubated at 30 °C for 60 min in 1 mg/ml collagenase (type V, Sigma) solution containing 137 mm NaCl, 5 mm KCl, 4 mm NaHCO3, 2 mm MgCl2, 5.5 mm glucose, 10 mm PIPES, pH 6.5, and 2 mg/ml bovine serum albumin (Sigma) with gently shaking. Finally, the dispersed cells were plated onto culture dishes (see below) in a density of 1.5 × 105 cells/cm2 and were incubated at 37 °C with 5% CO2 atmosphere. To promote dedifferentiation of SMCs, isolated SMCs were cultured in Dulbecco’s modified Eagle's medium supplemented with 10% fetal calf serum (FCS) on plastic culture dishes. We used SMCs cultured under these conditions for more than 1 week as dedifferentiated SMCs. To maintain the differentiated phenotype, SMCs were cultured in Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin and 5 μg/ml insulin on laminin-coated dishes.

We prepared vascular SMCs from chicken aorta by explant methods (30). To isolate explant-derived SMCs, the sortia media was minced into 1–2-mm segments and incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. The cells were routinely passaged at a 1:3 ratio. We used three-passaged cells as dedifferentiated vascular SMCs.

**Two-dimensional Gel Electrophoresis**—Two-dimensional gel electrophoresis was performed according to the previously described method (39) with some modifications. Cells were washed with phosphate-buffered saline and lysed in 9.5 M urea, 2% Triton X-100, and 5% 2-mercaptoethanol by sonication. The concentration of amphotrye (pH 4.0–
6.0) was adjusted to 2%, and then the samples were separated by isoelectric focusing. First dimensional gel was composed of 9.2 M urea, 4% acrylamide/bisacrylamide, 2% Triton X-100 and 2% amphotrye (pH 4.0–6.0). Isoelectrofocusing was first conducted at 500 V for 10 min, followed by at 750 V for 3.5 h and then at 1000 V for 2 h. The second dimension was SDS-PAGE with 17.5% acrylamide. Separated proteins were then transferred to nitrocellulose membranes and were detected by the ECL Western blotting detection kit (Amersham Corp.) using specific antibodies. TM311, anti-chicken gizzard TM monoclonal anti-
body (mAb), was purchased from Sigma. Anti-CaD polyclonal antibi-
odies were prepared as described elsewhere (3). We also used polyclonal TM antibodies against chicken gizzard TMs (40) in this study.

**Preparation of Tropomyosin and Actin-binding Assay**—The SMCs were washed with phosphate-buffered saline and were harvested in phosphate-buffered saline containing 1 μg/ml leupeptin and 0.1 mm diisopropyl fluorophosphate. The cells were pelleted by brief centrifugation and then sonicated in 20 mM Tris-HCl, pH 7.5, 1 mM NaCl, 10 mM dithiothreitol (DTT), 10 mm EGTA, 0.5 mm diisopropyl fluorophosphate, and 5 μg/ml leupeptin. The lysates were boiled for 5 min, followed by centrifugation at 100,000 × g for 30 min. The supernatants thus obtained were subjected to ammonium sulfate precipitation between 28 and 36%. The pellets were suspended in 10 mM potassium phosphate, pH 7.0, 1 mM NaCl, 1 mM DTT and diazylated against the same buffer whose pH was lowered to 4.7 by the addition of HCl. After centrifuga-
tion at 15,000 × g for 20 min, the pellets were resuspended in and diazylated against 20 mM imidazole-HCl, pH 7.2, 100 mM NaCl, 0.1 mM DTT, and 0.1 mM EGTA. The dialyates were centrifuged at 100,000 × g for 30 min. All manipulations described above were carried out at 4 °C. The clarified dialyates (crude TM fractions) were used for actin-binding assays. The crude TM fractions were incubated with rabbit skeletal muscle actin at 37 °C for 30 min. The buffer conditions were as follows: 20 mM imidazole-HCl, pH 7.2, 100 mM NaCl, 0.1 mM DTT, 0.1 mM EGTA, and 5 mM MgCl2. After centrifugation at 100,000 × g for 30 min, the sedimented actin and its associated proteins were dissolved in two-dimensional gel electrophoresis buffer and subjected to two-dimen-
sional gel electrophoresis.

**cDNA Cloning and Expression of TM Isoforms**—cDNA libraries of SMCs and of dedifferentiated SMCs were constructed in Agt11. The libraries were screened using TM311 mAb. Alternatively, we amplified partial cDNA fragments carrying α-TM exons 3, 4, and 5 or β-TM exons 3, 4, and 5 by the polymerase chain reaction (PCR) method. These cDNA fragments were also used as probes for screening of the cDNA libraries. Cloned cDNAs were sequenced.

Glutathione-S-transferase (GST) and truncated TM fusion proteins were produced in *Escherichia coli* and purified by glutathione-sepha-
rose using the GST Gene Fusion System (Pharmacia Biotech Inc.). Each of the truncated cDNAs carrying exon 1a of α-TM, exons 2b to 9d (containing the 3'-noncoding region) of α-TM, exon 1a of β-TM, exons 2 to 9d (containing the 3'-noncoding region) of β-TM, or exons 1b to 6b of α-TM, was fused to the GST gene in the pGEX-3X vector. Immunologi-
cal reactivities of TM311 mAb against fusion proteins were examined by Western blotting.

We constructed expression vectors carrying each of the cDNAs for TM isoforms downstream of the chicken β-actin promoter (pAct-vector). Expression vectors were transfected into dedifferentiated SMCs as described elsewhere (12), and the cell lysates were analyzed by two-
dimensional gel electrophoresis and Western blotting with TM311 mAb as described above.

**Immunofluorescence Microscopy**—Expression of α-SM actin in differ-
etiated and dedifferentiated SMCs and chick embryo fibroblasts (CEFs) was examined by indirect immunofluorescence microscopy as described previously (40). In this study, fixed and permeabilized cells were incubated with anti-α-SM actin monoclonal antibody (Sigma) and then incubated with fluorescein isothiocyanate-labeled anti-mouse IgG antibody and rhodamine-phalloidin.

**Northern Blotting**—Total cellular RNAs were isolated from SMCs using the ISOGEN (Nippongene). Ten μg of total RNAs at the indicated culture days were separated on 1.0% agarose-formaldehyde gels and then transferred to nylon membranes (Hybond-N, Amersham). The membranes were hybridized with respective TM and CaD isofor-
specific DNA probes under the conditions listed in Table I. After re-

| Table I Conditions of Northern blotting |
|----------------------------------------|
| Probes | Nucleotide positions (GenBank™ accession No.) | Labeling | Hybridization buffer/temperature | Washing buffer/temperature |
| α-TM/E2α | 128–147 of α-TM-SM (D87893) | γ-32P[ATP] | HB-A’/48 °C | WB-A’/52 °C |
| α-TM/E2b | 264–283 of α-TM-F1 (D87891) | γ-32P[ATP] | HB-A/52 °C | WB-A/52 °C |
| β-TM/E1α-E2 | 28–263 of β-TM-SM (K02446) | γ-32P[ATP] | HB-B’/42 °C | WB-B’/42 °C |
| CaD/E3a | 811–932 of h-CaD (M97522) | γ-32P[ATP] | HB-B/42 °C | WB-B/42 °C |
| CaD/E3a-E5 | 757–776 of h-CaD (M97522) | γ-32P[ATP] | HB-A/52 °C | WB-A’/52 °C |

*Antisense oligo-DNA was phosphorylated by [γ-32P]ATP using T4 polynucleotide kinase (Takara).

1. cDNA fragment was amplified by PCR, and antisense strand DNA was labeled by [α-32P]dCTP using BcaBEST DNA polymerase (Takara).

2. HB-A consists of 6 × SSC (20 × SSC consists of 100 mM NaCl and 100 mM trisodium citrate dihydrate), 0.5% SDS, 1 mg/ml polyvinylpyrrolidone, 1 mg/ml bovine serum albumin, and 100 μg/ml herring sperm DNA.

3. HB-B consists of HB-A plus 50% formamide.

4. WS-A consists of 6 × SSC and 0.1% SDS.

5. WB-A consists of 2 × SSC and 0.1% SDS.

6. WB-B consists of 2 × SSC and 0.1% SDS.
moval of the hybridized probes, the membranes were reused. To visualize 28 S rRNAs, membranes were stained with 0.02% methylene blue. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis—Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from Oligo(dT)15-primed single-stranded cDNAs were synthesized from.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis—Oligo(dT)15-primed single-stranded cDNAs were synthesized from 3 μg of total RNAs using RAV-2 reverse transcriptase (Takara). 95%, 95%, and 95% volume of heat-treated single-stranded cDNA mixtures were subjected to PCR using specific sets of primers (Table II) and Exo

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TABLE III

**Coordinate Expression of α-Tropomyosin and Caldesmon Isosforms**

| Primer set         | 5′-Primer | 3′-Primer | Cycle number |
|--------------------|-----------|-----------|--------------|
| α-TM-exon 2a       | 5′-TTCCCTGTCTGATTTGCGG-3′ | 5′-GAGAGGAGGCTGCTGCTCAGG-3′ | 24 |
| α-TM-exon 2b       | 5′-TTCCCTGTCTGATTTGCGG-3′ | 5′-GAGAGGAGGCTGCTGCTCAGG-3′ | 24 |
| α-TM-common        | 5′-GCTTGAGCTGTGACTGGAAGGCG-3′ | 5′-GCTTGAGCTTTTTCCTCTTGGGCTTTCTTCTCTTCCATCTTTTTC-3′ | 24 |
| h-CaD              | 5′-TTCCCTGTCTGATTTGCGG-3′ | 5′-GCTTGAGCTTTTTCCTCTTGGGCTTTCTTCTCTTCCATCTTTTTC-3′ | 24 |
| CaD-common         | 5′-TTCCCTGTCTGATTTGCGG-3′ | 5′-GCTTGAGCTTTTTCCTCTTGGGCTTTCTTCTCTTCCATCTTTTTC-3′ | 24 |

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**RESULTS**

**Expression Change of TM Isosforms during SMC Dedifferentiation**—It is well known that primary cultured SMCs under serum-stimulated conditions convert from a differentiated to dedifferentiated state. We examined the isoform changes of TM in primary cultured gizzard SMCs. Western blotting of precultured SMCs on two-dimensional gels with an anti-chicken gizzard TM monoclonal antibody, TM311 mAb, revealed two major spots (spots a and c in Fig. II, A). In 3-day cultured cells under serum-stimulated conditions, new spots (spots I and 2) were detected with a more acidic isoelectric point and faster migration than spots a and c, whereas the latter became faint (Fig. II, B). The relative staining intensities of spots I and 2 gradually increased in 7-day cultured cells in accordance with the decreases in spots a and c (Fig. II, C). During this process, the intensity of spot a reduced faster than that of spot c. In two passaged cells, spots I and 2 were the only TM isoforms detected by TM311 mAb (Fig. II, D).

In this study, we used TM311 mAb as one of anti-TM antibodies. Although the epitope of TM311 mAb has not been characterized, several reports have suggested it to be the antibody cross-reacting with high-Mα TM isoforms (40, 41). To identify the epitope, we constructed bacterial expression plasmids carrying fusion proteins between GST and truncated α- or β-TM isoforms; exon 1a of α-TM; exons 2b, 3, 4, 5, 6b, 7, 8, 9, and 9d of α-TM; exon 1a of β-TM; exons 2, 3, 4, 5, 6a, 7, 8, and 9d of β-TM; and exons 1b, 3, 4, 5, and 6b of α-TM. The TM311 mAb only cross-reacted with truncated proteins including exons 1a of both α- and β-TMs, but not with the other truncated proteins (Fig. 2). Since amino acid sequences encoded by exons 1a of the α- and β-TM genes are 87% identical, the TM311 mAb is believed to recognize the limited regions of the high Mα and β-TM isoforms encoded by the respective exons 1a.
Coordinate Expression of α-Tropomyosin and Caldesmon Isoforms

FIG. 1. Expressional change of TM isoforms during dedifferentiation of SMCs. A, cell lysates from precultured and cultured gizzard and vascular SMCs were analyzed by two-dimensional gel electrophoreses (left; basic; right; acidic) followed by Western blotting using TM311 mAb (A–D, H, and I). A, precultured SMCs; B, 3-day cultured SMCs; C, 7-day cultured SMCs; D, two-passaged gizzard SMCs; H, precultured SMCs; I, 7-day cultured vascular SMCs. TM isoforms detected by this antibody (spots a, c, i, and 2) and low M, TM isoforms (spots 3a and 3b) are illustrated (E). The identification of TM isoforms by actin-binding assay is shown (F and G). The TM fraction prepared from differentiated (F) and dedifferentiated gizzard SMCs (G) were cosedimented with rabbit skeletal muscle F-actin, and precipitated proteins were separated by twodimensional gel electrophoresis and stained by Coomassie Brilliant Blue. H, identification of TM isoforms by forced expression of cloned TM cDNAs (A–E). Cell lysates from dedifferentiated SMCs transfected with expression vectors carrying cloned TM cDNAs were analyzed by the same procedure as described above. A, vector only; B, SM-α-TM; C, SM-β-TM; D, α-TM-F1; E, α-TM-F2 cDNAs.

FIG. 2. Epitope mapping of TM311 mAb. GST (a) and GST-truncated TM fusion proteins such as GST-α-TM exon 1a (b), GST-α-TM exons 2b, 3, 4, 5, 6b, 7, 8, and 9d (c), GST-β-TM exon 1a (d), GST-β-TM exons 2, 3, 4, 5, 6a, 7, 8, and 9d (e), and GST-α-TM exons 1b, 3, 4, 5, and 6b (f) were expressed in E. coli. Purified fusion proteins were subjected to SDS-PAGE followed by Western blotting using anti-GST antiserum (A) or TM311 mAb (B).

finally identified by an actin-binding assay. These actin-binding proteins separated by two-dimensional gel electrophoresis were detected by Coomassie Brilliant Blue staining (Fig. II, F and G); spots a and c and spots I and 2 were major TM isoforms in both phenotypes of SMC. In dedifferentiated SMCs, faint spots (spots 3a and 3b) corresponding to low M, TM isoforms were observed by protein staining (Fig. II, G) and also by polyclonal TM antibodies against chicken gizzard TMs, which cross-reacted with both the high and low M, TM isoforms (data not shown). Since a similar two-dimensional gel pattern was obtained using the TM fractions without the actin-binding assay (data not shown), the low amounts of spots 3a and 3b in the total TM fractions were not due to differences in their affinities against actin. Thus, expression of low M, TM isoforms in SMCs was extremely low, suggesting that TM311 mAb can detect major TM isoforms expressed in SMCs.

There was a remarkable difference in expression of α-SM actin between dedifferentiated SMCs under serum-stimulated culture conditions and CEFs; α-SM actin was strongly expressed in dedifferentiated visceral SMCs (Fig. 3, B and E), whereas this isofrom in CEFs was a trace level (Fig. 3, A and F). α-SM actin was also undetectable in differentiated visceral SMCs (Fig. 3, A and D). The major actin isoform expressed in the cells is γ-actin as identified by immunoblotting and two-dimensional gel electrophoresis (data not shown). Based on immunostaining, we assessed the contamination of CEFs in cultured gizzard SMCs; 91 ± 6% of total 7-day cultured SMCs under serum-stimulated conditions were α-SM actin-positive (data not shown). This result suggests that the contamination of CEFs in cultured SMCs was less significant. Therefore, a change of TM isoform as presented here depends on phenotypic modulation of gizzard SMCs in culture but does not arise from replacement of contaminated fibroblasts derived from connective tissue and serosa.

We further analyzed isoform change of TM in vascular SMCs as associated with their phenotypic modulation (Fig. 1, H and I). In precultured vascular SMCs (differentiated SMCs), spots a and c were seen as major spots (Fig. 1, H and I). In contrast, in dedifferentiated vascular SMCs under serum-stimulated conditions, spots I and 2 were newly detected in place of spots a and c (Fig. 1, I). This isoform change of TM is consistent with that of TM during dedifferentiation of cultured gizzard SMCs (Fig. II, A–D). Thus, isoform interconversion of TM from spots a and c to spots I and 2 occurs in common with phenotypic modulation of both visceral and vascular SMCs.

Identification of TM Isoforms Whose Expression Is Dependent on SMC Phenotype.—To identify the major TM isoforms detected by TM311 mAb, we screened cDNA libraries of differentiated and dedifferentiated gizzard SMCs with TM311 mAb or specific α- or β-TM cDNA probes and obtained four different clones. Sequencing of two clones from differentiated SMCs revealed α-TM-SM (36) and β-TM-SM (42), and two other clones from dedifferentiated SMCs were α-TM-F1 and α-TM-F2 (36). These four clones belong to high M, TM isoforms including exon 1a. cDNAs encoding other high and low M, TM isoforms...
were not detected. Although the cDNA sequences of α-TM-F1, α-TM-F2, and α-TM-SM have been speculated upon from the genomic structure of the α-TM gene, the present results reveal the first sequence data of their TM isoforms (DDBJ/EMBL/GenBank™ data bank with accession numbers D87891–D87893).

To identify the four spots summarized in Fig. 1E, the TM cDNAs obtained were transfected in dedifferentiated gizzard SMCs. The cells transfected with α-TM-SM or β-TM-SM cDNAs showed newly intensive spots corresponding to spots a and c (Fig. 1I, B and C). Transfection of α-TM-F1 and α-TM-F2 cDNAs increased in spots 1 and 2, respectively (Fig. 1II, D and E). These results indicate that α-TM-SM and β-TM-SM (spots a and c) substitute for α-TM-F1 and α-TM-F2 (spots 1 and 2) during dedifferentiation of SMCs. Based on the present results, exon structures of the α- and β-TM genes and their alternative splicings for α-TM-F1 and α-TM-F2 are summarized in Fig. 4, A and B. A significant difference among α-TM-SM, α-TM-F1, and α-TM-F2 is the selection between exons 2a and 2b; exon 2a is specifically spliced in the mRNA for α-TM-SM in differentiated SMCs, whereas the mRNAs for α-TM-F1 and α-TM-F2 in dedifferentiated SMCs contain exon 2b, indicating that this exon selection is dependent on the SMC phenotype. In contrast, expression of β-TM-SM was down-regulated during SMC dedifferentiation.

Coordinate Expressional Changes of TM and CaD Isoforms in Association with Phenotypic Modulation of SMCs—It has been demonstrated that isoform interconversion of CaD is associated with phenotypic modulation of SMCs (1, 3, 4). In regard to the expression change of CaD isoforms, the mRNA for h-CaD, in which exons 3b and 4 were spliced, converted to the mRNA for h-CaD within 24 h after serum stimulation (Fig. 5F). The switching of CaD isoforms by alternative splicing was completely coincident with that of α-TM isoforms.

To further compare phenotype-dependent isoform interconversion of TM and CaD, we introduced a primary culture system maintaining a differentiated phenotype of SMCs cultured on laminin-coated dishes in the presence of insulin. The differentiated phenotype-specific expression of TM and CaD was maintained even in 10-day cultured SMCs under conditions whereby α-TM-SM, β-TM-SM, and h-CaD were continuously expressed (Fig. 5II). In contrast, serum and PDGF-BB induced the differentiated phenotype-specific expression of TM and CaD isoforms; α-TM-SM converted to α-TM-F1 and α-TM-F2, β-TM-SM was down-regulated, and h-CaD was synchronously changed to l-CaD (Fig. 5, III and IV). Thus, exon selection in the α-TM and CaD genes (exons 2a and 2b in the α-TM gene and exons 3a, 3ab, and 4 in the CaD gene) would be coordinately regulated in a SMC phenotype-dependent manner. We also characterized such exon selection in the TM and CaD genes by semiquantitative RT-PCR methods during dedifferentiation of SMCs by serum stimulation. Coordinate changes from exon 2a to exon 2b in the α-TM gene and from exons 3ab and 4 to exon 3a in the CaD gene were observed during dedifferentiation, and these exon conversions were completed in 8-day cultured SMCs (Fig. 6). These results are in good accord with the Northern blotting data (Fig. 5II). Laminin retarded such isoform interconversions induced by serum (Fig. 5, III and J). PDGF-BB was induced to down-regulate the mRNAs for α-TM-SM, β-TM-SM, and h-CaD, and this effect was more

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**Figure 3. Expression of α-SM actin in different phenotypes of gizzard SMCs and CEFs.** Differentiated gizzard SMCs (A and D), dedifferentiated gizzard SMCs under serum-stimulated culture conditions (B and E), and CEFs (C and F) were stained with rhodamine-phalloidin (A, B, and C) or anti-α-SM actin monoclonal antibody (D, E, and F). Gizzard SMCs started to express α-SM actin during phenotypic modulation. α-SM actin is only scarcely expressed in CEFs.
potent than that of serum (Fig. 5, III and IV). We analyzed cell numbers of SMC cultured on laminin under nonstimulated and serum- or PDGF-BB-stimulated conditions. Culture conditions were identical with those of Northern blotting analyses described above: gizzard SMCs were cultured on uncoated dishes in the presence of bovine serum albumin and insulin (II). At 3 days of culture, SMCs on laminin-coated dishes were stimulated by 10% FCS (III) or 20 ng/ml PDGF-BB (IV), and they were further cultured for 1–7 days under the same conditions (corresponding to 4–10 days of culture). Total RNAs were isolated from precultured gizzard SMCs (I) and cultured gizzard SMCs at the indicated days (days 3–10). Ten micrograms of RNAs were separated on formalin-agarose gels, transferred to nylon membranes, and hybridized with the indicated exon-specific DNA probes. 28S rRNAs were stained by methylene blue. Relative cell numbers of SMCs cultured on laminin under nonstimulated (circles) and serum-stimulated (squares), or PDGF-BB-stimulated (triangles) conditions are shown (V). Culture conditions were identical with those of Northern blotting analyses.

FIG. 5. Expression of alternatively spliced TM and CaD mRNAs during phenotypic modulation of SMCs. Gizzard SMCs were cultured on uncoated dishes under serum stimulation (I) and on laminin-coated dishes (II–IV) as described under “Materials and Methods.” The SMCs in the presence of 10% FCS were cultured on uncoated dishes (I). The cells were cultured on laminin-coated dishes in the presence of bovine serum albumin and insulin (II). At 3 days of culture, SMCs on laminin-coated dishes were stimulated by 10% FCS (III) or 20 ng/ml PDGF-BB (IV), and they were further cultured for 1–7 days under the same conditions (corresponding to 4–10 days of culture). Total RNAs were isolated from precultured gizzard SMCs (P) and cultured gizzard SMCs at the indicated days (days 3–10). Ten micrograms of RNAs were separated on formalin-agarose gels, transferred to nylon membranes, and hybridized with the indicated exon-specific DNA probes. 28S rRNAs were stained by methylene blue. Relative cell numbers of SMCs cultured on laminin under nonstimulated (circles) and serum-stimulated (squares), or PDGF-BB-stimulated (triangles) conditions are shown (V). Culture conditions were identical with those of Northern blotting analyses.

Coexpression of α-TM-SM and h-CaD mRNAs in Developing Chick Embryos—To analyze the expressional profiles of α-TM-SM and h-CaD in vivo, we carried out in situ hybridization in developing chick embryos. For this experiment, we used specific oligoprobes described under “Materials and Methods,” which specifically hybridized with the mRNAs for α-TM exon 2a, α-TM exon 2b, h-CaD, and l-CaD by Northern blotting (data not shown). Expressional profiles of the mRNAs for α-TM exon 2a, α-TM exon 2b, h-CaD, and l-CaD in 8–21-day-old chick embryos are shown in Fig. 7. The h-CaD transcripts were localized in tissues containing SMCs such as esophagus, crop,
FIG. 6. RT-PCR analysis of expressional changes of α-TM and CaD isoforms during dedifferentiation of SMCs under serum-stimulated culture conditions. Three micrograms of total RNAs prepared for Northern blotting (Fig. 5III) were subjected to RT-PCR (I). Oligo(dT)15-primed single-stranded cDNAs were used as PCR templates. PCRs were carried out under conditions summarized in Table II, and the products were stained with SYBR Green I. Relative contents of each mRNA were estimated by multiregression analysis as described under “Materials and Methods” (II). The relative ratios of α-TM mRNA containing exon 2a or exon 2b to total α-TM mRNAs and the ratios of high or low Mr CaD mRNA to total CaD mRNAs are shown. Open triangles, α-TM mRNA containing exon 2a/total α-TM mRNAs; closed triangles, α-TM mRNA containing exon 2b/total α-TM mRNAs; open circles, h-CaD mRNA/total CaD mRNAs; closed circles, l-CaD mRNA/total CaD mRNAs.

proventriculus, gizzard, intestine, lung, and aorta in developing embryos (Fig. 7C and 8A). As shown in Fig. 7, A and C, the localization of α-TM-SM transcripts coincided with that of h-CaD transcripts, and α-TM-SM transcripts were detectable in 8-day-old embryo. In contrast to h-CaD, l-CaD transcripts were weak and were ubiquitously distributed (Fig. 7D). The distribution of l-CaD mRNA in gizzard was opposite to that of h-CaD mRNA; h-CaD mRNA was highly expressed in the muscle layer, whereas l-CaD mRNA was restricted mainly to the inner layer of the lumen (Fig. 8B). The localization of α-TM-SM mRNA probed by an exon 2a-specific oligoprobe was shown to be identical to that of h-CaD mRNA (Fig. 7, A and C). Exon 2b in the α-TM gene is selected in almost all cell types except for differentiated SMCs. In 8-day-old embryo, α-TM isoforms containing exon 2b were evenly expressed in both smooth and skeletal muscles. During development, such expressions gradually increased in skeletal muscles but decreased in smooth muscles (Fig. 7B).

DISCUSSION

The SMCs display phenotypic modulation from a differentiated to dedifferentiated state under conventional culture conditions. During dedifferentiation, the morphology of SMC shows a dramatic change, from a long spindle-like to a proliferative fibroblast-like shape. In accordance with this process, h-CaD, which is a highly favorable parameter for the differentiated phenotype of SMCs, converts to l-CaD (43–45). On the other hand, a change of CaD isoforms from the low to high Mr form links to SMC differentiation (1, 3, 4). Isoform interconversion associated with phenotypic modulation of SMCs is also observed in other cytoskeletal and contractile proteins, actin (1, 2), myosin heavy chain (5, 6), and vinculin/meta-vinculin (1, 7). Here we have identified the epitope of TM311 mAb in the amino acid sequences encoded by exons 1a of α- and β-TM genes (Fig. 2) and used this antibody to investigate isoform interconversion of TMs in association with phenotypic modulation of SMCs. cDNA cloning of major TM isoforms from differentiated and dedifferentiated SMCs and identification by overexpression of isolated cDNAs revealed the presence of four TM isoforms: α-TM-SM changed to α-TM-F1 and α-TM-F2, and β-TM-SM was down-regulated during dedifferentiation (Fig. 1).

Since an identical change of TM isoforms was found in visceral and vascular SMCs, these molecular events were common to both phenotypes of SMC. Based on the difference of α-SM actin expression in dedifferentiated SMCs and CEFs (Fig. 3), the expressional change of TM isoforms is caused by phenotypic modulation of SMCs themselves, and not be due to the replacement of differentiated SMCs by contaminated fibroblasts.

It has been reported that isoform diversity of TM is caused by alternative splicing and differential promoter usage in the TM genes (32). Regarding the α-TM gene, exon 2a is specifically spliced in the α-TM-SM mRNA, and exon 2b is spliced in other α-TM isoform mRNAs. Selection between exons 2a and 2b is a critical event during phenotypic modulation of SMCs. As shown in Figs. 5 and 6, exon selection of the α-TM gene and that of the CaD gene might be coordinately regulated during dedifferentiation of SMCs induced by serum or PDGF-BB stimulation. Inversely, both the mRNAs for h-CaD and α-TM-SM were developmentally coexpressed in visceral and vascular smooth
day-old chick embryo. In situ distal 5' as an exon enhancer element, causing predominant selection of
been reported that repeating purine-rich motifs in exon 3b act
phenotype-dependent fashion. The SMC-specific splicing in the
SMC-specific suppression (38). The third possibility is that the
suppression of exon 2b selection; two conserved elements in
each of the introns flanking exon 2b are essential for such
exon selection (4.5 days) and increases during development, suggesting that
expression of α-TM-SM occurs at very early developmental
stages (54). However, these results are unclear with respect to
localization of TM isoform mRNAs, because such study has
been performed by RT-PCR using RNAs from whole embryoid
bodies and embryos. Here we have been the first to demon-
strate the localization and developmental changes of TM and

However, a different result was obtained in our separate ex-
periments, suggesting that the intron sequence between exons
3b and 4 might be involved in alternative selection of distal and
proximal 5'-splice sites within exon 3. At present, it remains
unclear whether common factor(s) are involved in such exon
selection in the α-TM and CaD genes. Further study will be
necessary to reveal the phenotype-dependent coordinate splic-
ing mechanism of these genes.

Compared with immediate changes in exon selection in the
α-TM and CaD genes during the dedifferentiation process, the
down-regulation of β-TM is moderate. These molecular events
are characteristic of SMC phenotype-dependent changes. While
transcriptional machineries of the TM genes have not been well
characterized, the upstream promoter of the β-TM gene has
been partially analyzed in a skeletal muscle cell line; a CarG
box-like motif was identified as one of the essential cis-ele-
ments necessary for skeletal muscle-specific transcription (47).
A CarG box-like motif was found in the 5'-upstream regions of
muscle-specific genes such as the α-skeletal (48) and α-SM
actin genes (8, 9), the CaD gene (12), the SM22 gene (21),
and the myosin heavy chain gene (9). In the case of the α-skeletal
actin gene, a CarG box-like motif functions as either a positive
or negative regulatory element; trans-acting factors (serum
response factor and YY1) bound to the CarG box-like motif may
be involved in positive and negative regulations, respectively
(49). Detailed promoter analysis of the β-TM gene including
transcription factor(s) is of future interest.

As shown in Figs. 1 and 5I, primary cultured SMCs by
serum-stimulation convert their phenotype from a differenti-
ated to dedifferentiated state using molecular markers such as
TM and CaD isoforms. Serum-derived growth factors and ex-
tracellular matrices such as fibronectin are known to promote
dedifferentiation of SMCs (50). In a separate experiment, we
found that laminin and insulin show a potent ability to main-
tain a differentiated phenotype of primary cultured SMCs.2 In
fact, laminin is abundant in extracellular matrices of both
aortic media and visceral muscle layers (51), and its expression
is developmentally regulated (52). It has also been reported
that laminin is involved in functional differentiation of mam-
mary gland epithelial cells to activate the β-lactoglobulin gene
(53). In regard to gene expression, such as the α- and β-TM and
CaD genes, these gene expressions were found in differentiated
SMCs only in the presence of laminin and insulin (Fig. 5J).
Despite the presence of laminin, PDGF-BB induced isoform
changes of α-TM-SM and h-CaD and down-regulation of β-TM-
SM with much more potency than serum (Fig. 5, III and IV).
However, the PDGF-BB did not promote cell proliferation (Fig.
5V). These results suggest that dedifferentiation of SMCs is
independent of cell growth.

There is no report investigating the experssional change of
TM isoforms associated with phenotypic modulation of SMCs.
Most recently, a developmental change of TM isoforms has
been demonstrated using mouse embryonic stem cells and em-
bryos; α-TM-SM mRNA is expressed in undifferentiated em-
byronic stem cells and in all stages of developing embryoid
bodies, whereas the mRNA is detectable in postcoitum embryo
(4.5 days) and increases during development, suggesting that
expression of α-TM-SM occurs at very early developmental
stages (54). However, these results are unclear with respect to
localization of TM isoform mRNAs, because such study has
been performed by RT-PCR using RNAs from whole embryoid
bodies and embryos. Here we have been the first to demon-
strate the localization and developmental changes of TM and

2 K. Hayashi, Y. Chimori, H. Saga, and K. Sobue, manuscript in
preparation.

Fig. 8. Localization of h-CaD (A) and l-CaD (B) mRNAs in 15-
day-old chick embryo. In situ hybridization using an h-CaD-specific
probe (A and B left) and a l-CaD probe (B, right) was performed. br, brain; ey, eye; es, esophagus; cr, crop; ao, aorta; he, heart; lu, lung; pr,
proventriculus; gi, gizzard; in, intestine.
Cd mRNA by in situ hybridization. The mRNAs for α-TM and h-CaD were confined to tissues containing SMCs and were closely coexpressed in each embryonic stage. Thus, expressions of α-TM-SM and h-CaD are coordinately regulated in vivo as well as in vitro. In conclusion, alternative splicing in exons 2a and 2b in the α-TM gene and exons 3 and 4 in the Cd gene might be controlled by a common mechanism that is closely associated with SMC phenotype.

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