Smooth Muscle Cell Phenotype-dependent Transcriptional Regulation of the α1 Integrin Gene*

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The expressional regulation of chicken α1 integrin in smooth muscle cells was studied. The α1 integrin mRNA was expressed developmentally and was distributed dominantly in vascular and visceral smooth muscles in chick embryos. In a primary culture of smooth muscle cells, α1 integrin expression was dramatically down-regulated during serum-induced dedifferentiation. Promoter analyses revealed that the 5′-upstream region (−516 to +281) was sufficient for transcriptional activation in differentiated smooth muscle cells but not in dedifferentiated smooth muscle cells or chick embryo fibroblasts. Like other α integrin promoters, the promoter region of the α1 integrin gene lacks TATA and CCAAT boxes and contains binding sites for AP1 and AP2. The essential difference from other α integrin promoters is the presence of a CArG box-like motif. Deletion and site-directed mutation analyses revealed that the CArG box-like motif was an essential cis-element for transcriptional activation in differentiated smooth muscle cells, whereas the binding sites for AP1 and AP2 were not. Using specific antibodies, a nuclear protein factor specifically bound to the CArG box-like motif was identified as serum response factor. These results indicate that α1 integrin expression in smooth muscle cells is regulated transcriptionally in a phenotype-dependent manner and that serum response factor binding plays a crucial role in this regulation.

Integrins, heterodimeric transmembrane proteins, consist of an α and a β subunit and play a role in cell-to-cell and cell-to-extracellular matrix adhesion as well as intracellular signal transduction (1). Cell adhesion mediated by integrins is important for cell differentiation, proliferation, and migration. There are at least 15 α and 8 β subunits, and their combinations generate various integrins that are distributed widely in a variety of cells. It has been generally accepted that the α subunit dictates ligand binding specificity and that the cytoplasmic domain of the β subunit, which interacts directly with the cytoskeleton, is involved in signal transduction from the extracellular matrix to the cytoplasm. Among variations of the integrin family, αβ1 integrin is a receptor for both laminin (2) and collagens (3). During quail embryogenesis, the expression of α1 integrin is curious; α1 integrin is expressed transiently in nervous tissues and skeletal and cardiac muscle cells of early embryos, whereas its expression is increased dramatically in smooth muscle tissues as development proceeds (4). At a late stage of embryogenesis and afterward, α1 integrin expression comes to be restricted in visceral and vascular smooth muscle cells (SMCs)1 and microvascular endothelium, whereas it is absent from most epithelial tissues. The down-regulation of α1 integrin expression has been reported in cultured SMCs under serum-stimulated conditions (5, 6) and in some leiomyosarcomas (7); therefore, α1 integrin expression is closely associated with a phenotype of SMC.

Although the SMC originates from neural crest and mesodermal precursors, the precise mechanisms of lineage and phenotypic modulation of SMCs remain unknown. Several cytoskeletal and contractile proteins have been used recently as molecular markers for an SMC lineage and phenotype. Among them, isoform changes of caldesmon (CaD) (8), myosin heavy chain (9), α-tropomyosin (10), and vinculin/meta-vinculin (11) are associated with phenotypic modulation of SMC. Their expressions are regulated by SMC phenotype-dependent splicings. On the other hand, expressions of smooth muscle α-actin (12), calponin (13), and SM22α (14) are regulated transcriptionally in an SMC phenotype-dependent manner (15); they are up-regulated in differentiated SMCs, but down-regulated in dedifferentiated SMCs. Whereas cis-element and trans-acting factor(s) of smooth muscle α-actin (16–18), CaD (19), myosin heavy chain (20), SM22α (14, 21), and calponin (22) genes have been reported in part, SMC-specific transcriptional machineries are not understood fully.

Transcriptional machineries of integrin genes such as α2 (23), α4 (24–26), CD11a (αL) (27), and CD11c (αX) (28) have been well studied. In general, the integrin promoters contain multiple Sp1, AP1, and/or AP2 binding sites, but not TATA and CAAT boxes, as cis-elements. In the case of α1 integrin, its expression depends upon a phenotype of SMC as described above. It is therefore worthwhile to investigate an SMC-specific transcriptional regulation of the α1 integrin promoter. In this study, we attempted to understand how α1 integrin expression is regulated in SMCs. As a first step, we cloned full-length cDNA encoding chicken α1 integrin and found that α1 integrin expression during phenotypic modulation of SMC is regulated

1 The abbreviations used are: SMC(s), smooth muscle cell(s); CaD, caldesmon; RACE, rapid amplification of cDNA end; PCR, polymerase chain reaction; kbp, kilobase pair(s); CEFs, chick embryo fibroblasts; SRF, serum response factor; bp, base pair(s); PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); CAT, chloramphenicol acetyltransferase; SKE, serum response element.

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Transcriptional Regulation of the α1 Integrin Gene in SMCs

Northern Blotting—Total RNAs were extracted from 15-day-old chick embryo gizzard, aortic medial muscle layers, and primarily cultured SMCs and chick embryo fibroblasts (CEF) using an ISOGENT RNA extraction kit (Nippon Gene, Japan). α1 integrin cDNA fragments, expanding from 575 to 1140 bp (Fig. 1A, probe N2), amplified from −177 to 27 (Fig. 1A, probe N2), were amplified by PCR, and they were 32P labeled on the antisense strands and used as probes. CaD cDNA (GenBank M28417) fragments (expanding from 286 to 810 for the probe common to h- and I-CaD) and expanding from 811 to 1438 for the probe specific to h-CaD), a calponin cDNA (GenBank M65595) fragment (expanding from 1 to 867), an SM22a cDNA (GenBank M53165) fragment (expanding from 99 to 524), and a serum response factor (SRF) cDNA (GenBank U50596) fragment (expanding from 30 to 803) were labeled in the same way. 2 µg of total RNAs were separated on 1.0% agarose-formaldehyde denaturing gels and then transferred to nylon membranes. The membranes were hybridized with the probe at 42 °C for 16 h in 50% formamide, 6 × SSC, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.5% SDS, and 0.1 mg/ml denatured herring sperm DNA. The blots were washed in decreasing SSC concentrations with a final concentration of 0.1 × SSC containing 0.1% SDS at 52 °C. To quantify the applied RNAs, ribosomal RNAs were stained with 0.02% methylene blue.

Primer Extension and S1 Mapping—Primer extension was performed using a 32P end-labeled antisense oligonucleotide, corresponding to the 5′-upstream sequence from +57 to +77 (see Fig. 5A, H00129), as a primer. The 32P-labeled primer (1 × 105 cpm) was annealed to 20 µg of total RNAs from 15-day-old chick embryo gizzards and was extended with Rouss-associated virus 2 reverse transcriptase (Takara Shuzo, Japan) for 1 h at 42 °C. The extended products were analyzed on 8% polyacrylamide-urea denaturing gels.

As a probe for S1 mapping, a 140-bp genomic DNA fragment was amplified by PCR using a 32P end-labeled antisense primer, H00129 (see Fig. 5A). Purified 32P-labeled probe (1 × 106 cpm) was hybridized to 25 µg of total RNAs from 15-day-old chick embryo gizzards for 16 h at 37 °C in hybridization buffer containing 40 mM PIPES, pH 6.5, 0.4 mM NaCl, 1 mM EDTA, and 10% formamide. The hybridized mixture was digested with S1 nuclease (Takara Shuzo, 200 units/ml) in a buffer containing 50 mM sodium acetate, pH 4.5, 250 mM NaCl, 4.5 mM ZnCl2, and 1 mg/ml denatured herring sperm DNA for 30 min at 37 °C. The protected products were analyzed as described for primer extension analysis.

Cell Culture, Transfection, and CAT Assay—Gizzard SMCs were isolated from 15-day-old chick embryos and were cultured in Dulbecco’s modified Eagle’s medium supplemented with 0.2% bovine serum albumin on laminin-coated dishes (10). Under such culture conditions, a differentiated phenotype of gizzard SMC was maintained for several days (see “Results”). The isolated SMCs were also cultured in Dulbeccco’s modified Eagle’s medium supplemented with 10% fetal calf serum for more than 1 week to promote dedifferentiation (see “Results”). We used gizzard SMCs cultured on laminin without serum and under serum-stimulated conditions for promoter analysis in differentiated and dedifferentiated SMCs, respectively. Vascular SMCs were isolated from chick aortic media by explant methods (33) and were cultured under serum-stimulated conditions for promoter analysis in differentiated and dedifferentiated SMCs, respectively. Vascular SMCs were isolated from chick aortic media by explant methods (33) and were cultured under serum-stimulated conditions for promoter analysis in differentiated and dedifferentiated SMCs, respectively. Smooth muscle cells (SMCs) were isolated from chick aortic media by explant methods (33) and were cultured under serum-stimulated conditions for promoter analysis in differentiated and dedifferentiated SMCs, respectively.

MATERIALS AND METHODS

cDNA and Genomic DNA Cloning—A cDNA library of gizzard SMCs from 15-day-old chick embryos was constructed in Agt11 and was screened by plaque hybridization with 32P-labeled probes derived from a partial cDNA fragment encoding the I-domain of chicken α1 integrin (GenBank U1041). To confirm the sequences of 5′- and 3′-portions, rapid amplification of cDNA end (RACE) was introduced. Genomic clones carrying the 5′-upstream regions of the chicken α1 integrin gene were isolated from a chicken genomic DNA library constructed in ADASHI (28) using a 32P-labeled cDNA subclone, C3b (Fig. 1A), as a probe. The sequences of cDNA and genomic clones were determined.

Expression of the Cloned Full-length α1 Integrin cDNA in Cultured SMCs—The full coding region of the α1 integrin cDNA was amplified by polymerase chain reaction (PCR) using a cDNA clone, C8-1 (approximately 5 kb in length) (Fig. 1A, as a template, and the amplified cDNA was inserted into the downstream of the SV40 promoter in pcDNA3 (30). The plasmid thus obtained was expressed transiently in cultured SMCs and chick embryo fibroblasts (CEF) using ISOGEN according to the manufacturer’s recommended procedure. CAT assays were carried out as described previously (19, 29) with some modifications. Briefly, cells were plated on six-well culture dishes at a density of approximately 1 × 105 cells/well for differentiated gizzard SMCs or approximately 5.4 × 104 cells/well for the others to make an equal adhered cell density and were all transiently transfected using TransIT-LT1 polyamine transfection reagents (Pan Vera Corporation). CAT assays were performed with the same conditions as described previously. The sequences of cDNA and genomic clones were determined from chick embryo gizzards and was digested with the indicated restriction enzymes. The DNA fragments were separated on 0.7% agarose gels and transferred to a nylon membrane (Hybond-N+, Amersham) according to the method of Southern. A 32P-labeled DNA fragment, expanding from +103 to +306 in the 5′-upstream sequence (see Fig. 4A, probe E1), was used as a probe. Hybridization and washing were carried out under

at the mRNA level. We further characterized the 5′-upstream region of the α1 integrin gene and identified a CArG box-like motif as a cis-element involved in the SMC phenotype-dependent transcriptional regulation.

FIG. 1. AcDNA cloning of chicken α1 integrin. Panel A, alignment of maps of chicken α1 integrin cDNA and overlapping cDNA clones are shown schematically. Signal peptides and the I-domain are indicated by hatched and closed boxes, respectively. Nucleotide sequence of the coding region is numbered (1–3513). Panel B, homology plots of deduced amino acid sequences of chicken versus rat or human α1 integrins. Panel C, forced expression of α1 integrin. Eukaryotic expression vector carrying the full-length of the α1 integrin coding sequence was expressed transiently in dedifferentiated gizzard SMCs, and the cell lysates were analyzed by Western blotting using polyclonal antibodies against chicken α1 integrin: lane 1, no transfection; lane 2, vector plasmid; lane 3, vector plasmid carrying the α1 integrin cDNA; lane 4, differentiated gizzard SMCs.
the cells in Opti-MEM (Life Technologies, Inc.). After a further 4-h incubation, the medium was replaced with fresh culture medium, and the transfected cells were harvested 48 h later. Standardization of transfection efficiency was carried out using luciferase activity as described elsewhere (19, 34). Cell extracts containing equal amounts of luciferase activity were used for CAT assay. The transfection experiments were repeated at least three times on duplicate cultures with two or three different plasmid preparations. The CAT activity was quantified by Scanning Imager (Molecular Dynamics).

Analysis of DNA-Protein Interaction by Gel Shift Assay—Probes used for these analyses are shown in Fig. 7A. Nuclear extracts from differentiated and dedifferentiated gizzard SMCs were prepared as described elsewhere (35). Whole cell extracts from chicken aortic tissue and cultured vascular SMCs by explant methods were prepared according to the method described elsewhere (36). For characterization of DNA-protein interaction, samples of nuclear extracts (4 μg) were mixed with 0.1–0.2 pmol of 32P-labeled probe (see Fig. 7A) and 2 μg of denatured herring sperm DNA in the presence or absence of nonradiolabeled competitor in 20 μl containing 5 mM HEPEs, pH 7.8, 5 mM 2-mercaptoethanol, 1 mM EDTA, 60 mM NaCl, 5 mM spermidine, and 10% glycerol at room temperature for 20 min. Samples were analyzed on 5% polyacrylamide gels in 0.5 × Tris borate-EDTA (TBE) buffer. Anti-SRF polyclonal antibodies were purchased from Santa Cruz Biotechnology. We confirmed the cross-reactivity of these antibodies to chicken SRF (data not shown).

In Situ Hybridization of Chick Embryos—Chick embryos at various stages were frozen and sectioned 12–15 μm thick by cryostat (Bright, U. K.). The sections were mounted on polysilane-coated glass slides and fixed with 4% formaldehyde in 0.1 M phosphate buffer for 20 min followed by treatment with 10 μg/ml proteinase K and acetylation with 0.25% acetic anhydride and 0.1 M triethanolamine. The slides were washed in 0.1 M phosphate buffer and dehydrated in ethanol. A subfragment of chicken α1 integrin cDNA, expanding from 1137 to 1835 (Fig. 1A, probe 1) and exon 3b of the chicken CaD gene were subcloned into pGEM-4Z vector (Promega). Radiolabeled cRNA probes were prepared using T7 RNA polymerase (Promega) and [α-32P]UTP (NEN Life Science Products) and purified with a CHROMASPIN-100 column (Clontech). Hybridization was performed at 55 °C in 20 mM Tris-citrate, pH 8.0, 10 mM phosphate buffer containing 1 mM EDTA, 0.3 M NaCl, 5% formamide, 1× Denhardt’s solution, 0.2% Sarcosyl, 500 μg/ml yeast tRNA, 200 μg/ml herring sperm DNA, and the labeled probe (2–3 × 106 cpm). Next, a high stringency wash was performed in 2× SSC, 5% 2-mercaptoethanol, and 50% formamide for 30 min at 65 °C. The remaining probes were digested with RNase A treatment, and an additional high stringency wash was carried out. Finally, the slides were sequentially dehydrated in ethanol and exposed to Fuji PhosphorImaging plate. Data were collected and analyzed with BAS-5000 PhosphorImager (Fujiﬁlm, Japan).

RESULTS
cDNA Cloning of Chicken α1 Integrin—To isolate full-length cDNA, a partial cDNA fragment of chicken α1 integrin encoding the I-domain was amplified by PCR and was used as a probe for screening. Six overlapping cDNA clones (Fig. 1A) were isolated. Sequence analyses revealed an open reading frame of full-length chicken α1 integrin cDNA. The 5′- and 3′-untranslated cDNA sequences were also cloned by RACE as described under “Materials and Methods.” The cDNA fragment obtained by the 3′-RACE was terminated at the d(A)30 sequence in the 3′-untranslated region, but a polyadenylation signal is absent within the preceding 50 bp (Fig. 1A). As shown in Fig. 1A, both overlapping sequences cloned by plaque hybridization and RACE were identical. A sequence surrounding the ATG triplet for initiating methionine agreed well with the consensus sequence for functional translation initiating codons in eukaryotic mRNAs (37). The amino acid sequence of chicken α1 integrin showed about 65% identity with that of rat (38) or human (39) α1 integrin (Fig. 1B). However, the sequence of predicted signal peptides was not homologous. Using a cDNA probe carrying the 5′-untranslated region and the signal peptide coding region (probe N2 in Fig. 1A), the hybridization pattern observed was the same as that using the cDNA probe belonging to the homologous region (probe N1 in Fig. 1A) by Northern blotting (see Fig. 3B and data not shown). We further constructed an expression plasmid carrying the full-length open reading frame in eukaryotic expression vector and transiently expressed in dedifferentiated gizzard SMCs in which the endogenous α1 integrin was scarcely detectable (Fig. 1C). Western blotting analysis with anti-chicken α1 integrin polyclonal antibodies revealed that the transfected cells expressed a 175-kDa protein, and its migrating position in SDS gels was identical to that of endogenous α1 integrin in differentiated gizzard SMCs (Fig. 1C). These results indicate that cloned cDNAs certainly encode chicken α1 integrin.

Distribution of α1 Integrin mRNA in Developing Chick Embryos—It has been reported by immunohistological study that α1 integrin is expressed widely in early embryos and comes to be restricted in smooth muscles and endothelium thereafter (4). However, there is no assessment of mRNA expression of α1 integrin. We examined the distribution of α1 integrin mRNA in developing chick embryos (8-day-old to post-hatched) (P.H.) represented. Abbreviations: ao, aorta; cr, crop; es, esophagus; ge, gizzard; in, intestine; kd, kidney; ln, lung; and pr, proventriculus.

**Fig. 2. Distribution of the α1 integrin mRNA in developing chick embryos.** In situ hybridization was performed using riboprobes of the α1 integrin (panel A) and h-CaD (panel B). Sagittal sections through 8 (E8), 12 (E12), and 15 (E15)-day-old chick embryos and post-hatched chick (P.H.) are represented. Abbreviations: ao, aorta; cr, crop; es, esophagus; ge, gizzard; in, intestine; kd, kidney; ln, lung; and pr, proventriculus.
and 2). In our separate experiment, we have found that differentiat-
eted phenotype-specific isoforms of tropomyosin, a-TM-SM and
b-TM-SM, were expressed dominantly in such primary
cultured SMCs (41). During this transition, the expression of
a integrin (data not shown). These results suggest
contrast, the expression of a1 integrin mRNAs decreased dra-
matically during serum-induced dedifferentiation of gizzard
and vascular SMCs (Fig. 3B, lanes 3 and 5) and was faint in
CEF’s (Fig. 3B, lane 6). We analyzed the growth of SMCs
cultured on laminin under nonstimulated and serum-stimu-
lated conditions and investigated whether the expression pattern of such molecular markers was affected by the growth
state of cells or cell density. A dramatic growth of SMCs was
observed under serum-stimulated conditions, whereas signif-
cant cell growth was not found under nonstimulated condi-
tions. However, once a phenotype of SMCs was converted to the
dedifferentiated state, the cells were not able to recover the
expression of differentiation-specific markers even though they
were placed in a quiescent state by serum starvation (data not
shown) or contact inhibition in the postconfluent culture (10).
Thus, this phenotypic conversion is irreversible. It was the
same with a1 integrin (data not shown). These results suggest
that the expression of a1 integrin would be transcriptionally
regulated in an SMC phenotype-dependent manner.

Identification of Promoter Region of a1 Integrin Gene—To
isolate the 5'-upstream region of the a1 integrin gene, we
screened a chicken genomic library using a cloned cDNA frag-
ment. As a result of positive clone mapping, a 3.4-kbp EcoRI
fragment was hybridized with an oligonucleotide probe G1
(corresponding from +161 to +178 in the 5'-upstream sequence
(see Fig. 5A)). Sequencing analysis revealed that this fragment
contained approximately 2.5 kbp of 5'-flanking region, exon 1, and
part of intron 1 (Fig. 4A). Genomic Southern blotting using
probe E1 (Fig. 4A) resulted in a single hybridized band (Fig.
4B), suggesting that the a1 integrin is a single copy gene. A
part of the 5'-upstream sequence is shown in Fig. 5A. We then

FIG. 3. Expressional change of SMC-specific markers during
phenotypic modulation of cultured SMCs (A) and phenotype-de-
pendent expression of the a1 integrin mRNA in cultured SMCs
(B). The upper five panels in A and the top panel in B show Northern
blotting of total RNA samples isolated from the following cells or tissue:
in A, lane 1, precultured gizzard SMCs from 15-day-old chick embryo;
lane 2, 4-day cultured gizzard SMCs on a laminin-coated plate (differ-
etiated SMCs); lane 3, three time-passaged gizzard SMCs under se-
rum-stimulated conditions (dedifferentiated SMCs); in B, lanes 1–3,
same as A; lane 4, aortic tissue from 15-day-old chick embryo; lane 5,
three time-passaged vascular SMCs under serum-stimulated conditions
(dedifferentiated vascular SMCs); and lane 6, CEFs. 2 μg of RNAs were
separated on formalin-agarose gels, transferred to nylon membranes,
and the blots were hybridized with CaD cDNA probe encoding the
common sequence of h- and l-CaD (A, top panel); the locations of h-CaD
(4.8 kbp) and l-CaD (4.1 kbp) are indicated to the right of the blot, CaD
cDNA probe specific to h-CaD (A, second panel); calponin cDNA probe
(A, third panel); 1.8 kbp), SM22 cDNA probe (A, fourth panel; 1.2 kbp,
SRF cDNA probe (A, fifth panel; 3.6 kbp and 2.7 kbp), or a1 integrin
cDNA probe (B; 9.0 kbp and 4.5 kbp) as described under “Materials and
Methods.” In A, the same blot was used repeatedly after deprobing. The
bottom panels in both A and B show the 28 S rRNAs stained by meth-
ylene blue.

FIG. 4. Genomic structure of the 5'-upstream region of the a1
integrin gene. Panel A, restriction map of the 5'-upstream region of
the a1 integrin gene. The cutting sites of restriction enzymes and the
starting site of transcription (+1 with arrow) are indicated by the top
line: E, EcoRI; H, HindIII; P, PstI; and S, SmaI, respectively. The
exon/intron structure is shown schematically under the restriction map.
Open and shaded boxes indicate the first exon (Exon 1); the open box is
the 5'-untranslated region, and the shaded box is the coding region of
the a1 integrin. Thin line indicates the 5'-upstream region and the first
intron (Intron 1). Panel B, genomic Southern blotting of the a1 integrin
gene. 10 μg of chicken genomic DNA was digested with the indicated
restriction enzymes, separated by agarose gel electrophoresis, and
transferred to nylon membrane. The blots were hybridized with the
probe indicated in panel A (probe E1).
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**Fig. 5.** The nucleotide sequence of the 5'-upstream region of the α1 integrin gene (panel A) and identification of the transcription starting sites by S1 mapping (panel B). Panel A, major and minor transcription starting sites determined by S1 mapping are marked by large and small bent arrows, respectively. Dotted characters are the transcription starting sites determined by primer extension using the end-labeled antisense primer, HO0129. Bases are numbered (indicated in the left margin) with respect to the major starting site of transcription by S1 mapping. The sequences of cis-elements indicated with bold letters are underlined. Two binding sites for APIA and AP1B, respectively. Bold ATG triplets are the codon for initiating methionine of the α1 integrin protein. A 140-bp DNA fragment used as an S1 mapping probe is underlined. The corresponding sequences of antisense primers, HO0129, and probe G1, are shown by long horizontal arrows. Panel B, the 5'-end of antisense strand of the 140-bp DNA fragment indicated in panel A was specifically 32P-end labeled and was used for S1 mapping as described under "Materials and Methods." Lane 1, protected products; and lane 2, undigested probe. The positions of major and minor protected products (lane 1) are indicated in the left margin. For size standard, the 5'-end of antisense strand of the 257 bp DNA fragment indicated in panel B is shown by long horizontal arrows.
and a CArG box-like motif are present (Fig. 5A). To evaluate the contribution of these cis-elements to the transcriptional activity, we introduced mutations in each element and assessed the transcriptional activity of the respective mutated constructs in differentiated gizzard SMCs (Fig. 6, A and B). In comparison with the wild type construct (α1g5+CAT), CArG mutant (CArGMUT) exhibited a more than 10-fold decreased activity, whereas the transcriptional activity was not affected by the mutation of each AP1 site (AP1A and AP1AMUT) or AP2 site (AP2MUT) (Fig. 6B). The effect of mutation in the CArG box-like motif was also examined in dedifferentiated gizzard SMCs, vascular SMCs derived from aortic media explants, and CEFs. No significant change in the transcriptional activities was observed in these cells (Fig. 6B). These findings suggest that the CArG box-like motif would play an essential role in differentiated SMC-specific transcription of the α1 integrin gene.

**SRF Is a Core Factor Interacting with the CArG Box-like Motif**—To investigate interaction between the CArG box-like motif and nuclear proteins, we performed gel shift assays using a 100-fold excess of unlabeled α1CArGMUT without a competitor (lanes 1, 4, 7, and 10) or with a 100-fold excess of unlabeled α1CArGMUT (lanes 2, 5, 8, and 11) or with a 100-fold excess of unlabeled α1CArGMUT (lanes 3, 6, 9, and 12). Reaction mixtures were separated on 5% polyacrylamide gels. The shifted complexes are indicated by an arrowhead.

![FIG. 7](image)

**Fig. 7. A nuclear protein factor binds to the CArG box-like motif.** A, the top column shows the CArG box-like motif (enclosed in a rectangle) and its flanking DNA sequence in the α1 integrin promoter region. The middle panel shows a 22-bp double-stranded DNA sequence containing the CArG box-like motif. It was designated α1CArGMUT, and used as a probe or a competitor in gel shift assays. The bottom panel shows a mutant DNA sequence derived from α1CArG(22). It was designated α1CArGMUT and used as a competitor in gel shift assays. The replaced nucleotides in α1CArGMUT are indicated by dotted letters. B, gel shift assays revealed a specific complex between the CArG box-like motif and nuclear protein factors from SMCs. 4 μg of nuclear extract from gizzard SMCs from 15-day-old chick embryo (differentiated gizzard SMCs; lanes 1–3), three time-passaged gizzard SMCs under serum-stimulated conditions (dedifferentiated gizzard SMCs; lanes 4–6), adult aortic tissue (differentiated vascular SMCs, lanes 7–9), or three time-passaged vascular SMCs under serum-stimulated conditions (dedifferentiated vascular SMCs; lanes 10–12) were reacted with 32P-end-labeled α1CArG(22) without a competitor (lanes 1, 4, 7, and 10) or with a 100-fold excess of unlabeled α1CArG(22) (lanes 2, 5, 8, and 11) or with a 100-fold excess of unlabeled α1CArGMUT (lanes 3, 6, 9, and 12). Reaction mixtures were separated on 5% polyacrylamide gels. The shifted complexes are indicated by an arrowhead.
interaction was specific for the CArG box-like motif because the radiolabeled complex was efficiently replaced by an excess amount (100-fold) of unlabeled \(1\text{CArG}_{22}\), but not unlabeled \(1\text{CArG}_{22}\text{GMUT}\) in which the CArG box-like motif was replaced (Fig. 7, A and lanes 2 and 3 in B). The specific DNA-protein interactions observed in these assays were consistent with the transcriptional activities of \(\alpha 1\) integrin promoter in differentiated and dedifferentiated SMCs. Similar results were also obtained using nuclear extracts from vascular SMCs (Fig. 7B, lanes 7–12). The intensity of radiolabeled \(1\text{CArG}_{22}\)-protein complex in nuclear extracts from aortic tissue was stronger than that in nuclear extracts from dedifferentiated vascular SMCs. These results suggest that the interaction between \(1\text{CArG}_{22}\) and a protein factor depends on phenotypes of both visceral and vascular SMCs. It is well known that the serum response element (SRE) in \(c\text{-fos}\) promoter contains a CArG box motif, and SRF binds to this motif (42). The radiolabeled \(1\text{CArG}_{22}\)-protein complex competed efficiently with \(c\text{-fos}\) SRE (data not shown), suggesting the involvement of SRF in such a complex. To confirm the binding of SRF to the CArG box-like motif, we carried out supershift assay using anti-SRF antibody. As shown in Fig. 8, anti-SRF antibodies supershifted the \(1\text{CArG}_{22}\)-protein complex in a dose-dependent manner, but nonimmune IgG did not. This protein factor, in both nuclear extracts from gizzard and vascular SMCs, was recognized by the anti-SRF antibodies. This finding suggests that, at least in part, SRF is a core factor to interact with the CArG box-like motif and is a key factor for transcription of the \(\alpha 1\) integrin gene in both SMCs.

To investigate whether the expression of SRF is down-regulated during dedifferentiation of SMCs, we performed Northern blotting. As shown in Fig. 3A, the SRF mRNA did not decrease as drastically as the SRF binding activity in the gel shift assay did. This result suggests the presence of additional factor(s) that regulate the SRF binding in an SMC phenotype-dependent manner.

**Discussion**

Phenotypic modulation of SMC is associated with atherosclerosis and SMC-derived tumor progression. The SMCs in primary culture under serum-stimulated conditions display phenotypic modulation from the differentiated to dedifferentiated state. During this process the expression of some cytoskeletal proteins is altered dramatically, and these alterations are regulated at mRNA levels. Such expression changes of cytoskeletal proteins are used for favorable molecular markers indicating the SMC phenotype. Expression of \(\alpha 1\) integrin in SMCs has been reported to be up-regulated during embryonic development (4). In this study, we focused on the expression of \(\alpha 1\) integrin in association with an SMC phenotype and analyzed the transcriptional machinery of this gene using both differentiated and dedifferentiated phenotypes of SMCs.

We first cloned the full-length cDNA encoding chick \(\alpha 1\) integrin and the genomic DNA carrying the 5' upstream region of the \(\alpha 1\) integrin gene. The NH2-terminal half (extracellular domain) corresponding to the I-domain and seven repeated domains, the transmembrane domain, and the COOH-terminal cytoplasmatic domain of chicken \(\alpha 1\) integrin are highly homologous with those of rat (38) and human (39) \(\alpha 1\) integrins, although homology is not evident among the sequences corresponding to signal peptides (Fig. 1B). These results suggest that the domains involved in the function of \(\alpha 1\) integrin are highly conserved among different species. The reliability of our cDNA clone was confirmed by immunoreactivity of the protein produced by forced expression of the cloned cDNA against the anti-chicken \(\alpha 1\) integrin antibodies (Fig. 1C).

*In situ* hybridization revealed that the \(\alpha 1\) integrin mRNA was expressed predominantly in vascular and visceral smooth muscles in late stages of chick embryo. It was clearly detected in 8-day-old embryo and thereafter increased gradually in proportion to the expression of \(\text{h-CaD}\) mRNA (Fig. 2). Such an expression profile of the \(\alpha 1\) integrin mRNA almost completely coincided with its protein level in developing embryos (4). In contrast, the \(\alpha 1\) integrin was down-regulated dramatically in cultured SMCs during serum-stimulated dedifferentiation (Fig. 3B). These results indicate that the SMC phenotype-dependent expression of the \(\alpha 1\) integrin gene is regulated transcriptionally and that \(\alpha 1\) integrin is available as a molecular marker of SMC differentiation. In addition to SMCs, a low but a significant amount of \(\alpha 1\) integrin mRNA was detected in the kidney (Fig. 2). Korhonen et al. (43) also demonstrated the expression of \(\alpha 1\) integrin protein in mesangial and endothelial cells in fetal and adult human kidney.
The 5' upstream sequences of the α1 integrin gene share structural similarities with other α integrin genes (Fig. 5A); both TATA and CAAT boxes are absent, and consensus binding sites for AP1 and AP2 are present (23–28). It is still difficult to perform promoter analysis using differentiated vascular SMCs because no one has succeeded in preparing cultured vascular SMCs in a differentiated phenotype. We have established a primary culture system maintaining a differentiated phenotype of gizzard SMCs. Using our culture system, we analyzed the phenotype-dependent transcriptional regulation of the α1 integrin gene. The comparative analysis of a series of deleted α1 integrin promoter-CAT constructs revealed that the basal promoter activity of the gene was attributed to the region from +77 to +173 and that the 5' upstream sequence (−516 to +281) produced sufficient transcriptional activity (more than 10-fold activity compared with the basal activity) in differentiated gizzard SMCs (Fig. 6B). However, the transcriptional activities of the same sequence in dedifferentiated gizzard and vascular SMCs were much lower than that in differentiated gizzard SMCs (Fig. 6B). Site-directed mutagenesis revealed that the CArG box-like motif was important for the transcriptional activation in differentiated SMCs, whereas binding sites for AP1 or AP2 were not (Fig. 6B). Moreover, mutagenesis on the CArG box-like motif did not affect the transcriptional activities in both dedifferentiated SMCs and CEFs. These findings indicate that the CArG box-like motif serves as a positive element only in a differentiated phenotype of SMCs (Fig. 6B). The CArG box is known to be involved in quite different facets of the transcriptional regulation. One is the serum response of immediate early genes, mediated by SRE that has been initially identified in the c-fos proto-oncogene promoter (42). Another is the transcriptional activation of muscle-specific genes such as the skeletal and cardiac α-actin genes (44, 45) and the dystrophin gene (46). The involvement of the CArG box in gene expression has been emerging not only in striated muscle cells but also in SMCs. Promoter analyses of the smooth muscle α-actin (17, 18), smooth muscle myosin heavy chain (20), and Cd (19) genes have proven that the CArG box plays an essential role in SMC-specific transcription. The promoter region of the SM22α gene which directs tissue-specific expression in arterial SMCs in transgenic mice contains two CArG boxes (47, 48). It has been reported that SRF binds to the CArG boxes in the skeletal, cardiac, and smooth muscle α-actin promoters (18). Recently, Soulez et al. (49) directly showed that SRF is necessary for the CArG box-dependent transcriptional activation of muscle-specific genes. We demonstrated by supershift assays using anti-SRF antibodies that SRF is involved in the specific complex formation between the α1 integrin CArG box-like motif and SMC nuclear factors (Fig. 8). This complex formation was intensive when nuclear extracts from differentiated gizzard or vascular SMCs were used, whereas only faint complex formation was observed using nuclear extracts from both dedifferentiated SMCs (Fig. 7). Taken together, we first clarified that the CArG box-like motif in the α1 integrin promoter is a core element for SRF binding and that its binding is essential for the phenotype-dependent transcriptional regulation in SMCs. There was little difference in SRF expression between differentiated and dedifferentiated gizzard SMCs, whereas the binding activity of SRF to the CArG box-like motif in differentiated SMCs was much higher than that in dedifferentiated SMCs. This suggests the possible involvement of additional factor(s) that modulates the SRF binding to the CArG box-like motif. The CArG boxes have been shown to be responsible for SMC-specific gene expression as described above. Indeed, the CArG box-like motif in the α1 integrin gene may be replaceable with the CArG box sequence from another gene because gel shift assays revealed that the SRF binding to it competed not only with c-fos SRE but also with the CArG box in the Cd gene (data not shown). Despite the consensus core sequences of the CArG boxes and the CArG box-like motifs, there was no sequential homology surrounding these core sequences among SMC-specific genes, suggesting that the surrounding sequences are not essential for SMC-specific expression. SRF is distributed widely in all types of muscle tissues and other non-muscle cells (50). Therefore, SMC-specific transcriptional activation of the α1 integrin and other genes cannot be explained simply by SRF transactivation through its binding to the CArG box-like motif or the CArG box. Homeodomain proteins such as Phox/Mhou (51) or Nkx-2.5 (52) have been known to enhance the binding activity of SRF to CArG box and the transcriptional activity mediated by its binding. Considering these evidences, SMC-specific homeodomain proteins might be a potent candidate responsible for SMC phenotype-dependent and/or SMC-specific transcriptional regulations.

In summary, the present report is a first analysis of the α1 integrin promoter and has demonstrated that the interaction between the CArG box-like motif and SRF is essential for SMC phenotype-dependent transcriptional regulation. Further studies regarding a possible involvement of SMC-specific homeodomain proteins in the SMC-specific gene expression are necessary for elucidating the molecular mechanism of phenotypic modulation of SMCs.

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