Molecular Cloning and Biological Activity of a Novel Ha-Ras Suppressor Gene Predominantly Expressed in Skeletal Muscle, Heart, Brain, and Bone Marrow by Differential Display Using Clonal Mouse EC Cells, ATDC5*

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The mammalian ras protooncogenes, Ha-, Ki-, and N-ras, are expressed in a variety of tissues (1). For example, Ha-ras is highly expressed in skin and skeletal muscle; Ki-ras in gut and thymus; and N-ras in testis and thymus. Ras proteins bind guanine nucleotides and possess intrinsic GTPase activity, serving as transducers of diverse physiological signals including those controlling cellular proliferation and differentiation (2). Some of the biological activities of Ras proteins are known to be modulated by other proteins, including Krev-1 (3) and Ha-rev107 (4). However, physiological functions of Ras proteins and these regulatory proteins in mammalian cells remain largely unknown.

Chondrogenesis is a key event in skeletal development in vertebrates. We previously reported that chondrogenesis could be induced in chondroprogenitor-like EC cells, ATDC5, at a high incidence when cultured in the presence of insulin and TGF-β1, that ATDC5 cells keep track of the overt chondrogenesis *in vitro* (5–9). Clonal mouse embryonic fibroblast cells, C3H10T1/2, retain the properties of pluripotent mesodermal progenitors and have been shown to differentiate into adipocytes, myoblasts, osteoblasts, as well as chondrocytes (10) under distinct cultural conditions, including the presence of 5-azacytidine or high dose bone morphogenetic protein-2 (10, 11).

In this study, comparison by differential display of mRNAs expressed in undifferentiated ATDC5 cells with those in undifferentiated C3H10T1/2 cells led us to isolate a novel cDNA clone encoding a Ha-rev107-related protein predominantly expressed in skeletal muscle, heart, hippocampus, and bone marrow as well as ATDC5 cells.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions—**ATDC5 cells were plated in six-multitwell plates at an initial cell density of 6 × 10^4^ cells/well and cultured as described previously (5, 6). Clonal mouse embryonic fibroblast C3H10T1/2 cells (10), clonal mouse fibroblast NIH3T3 cells (13), clonal mouse fibroblast Balb/c 3T3 cells (14), and clonal mouse myoblast C2C12 cells (15) (RIKEN Cell Bank, Tsukuba, Japan) were plated in six-multitwell plates at an initial cell density of 6 × 10^4^ cells/well and cultured for 3 days in DMEM containing 10% fetal bovine serum (FBS). Clonal mouse newborn calvaria-derived osteogenic MC3T3-E1 cells (16) and clonal mouse stromal ST2 cells (17) were plated in six-multitwell plates at an initial cell density of 6 × 10^4^ cells/well and cultured for 3 days in α minimal essential medium containing 10% FBS. Cultured adherent cells from bone marrow were prepared from 6-week-old ICR mice (SRL, Hamamatsu, Japan) as described previously (18) with some modifications. Briefly, tibiae and femurs were dissected, the ends of the bones were cut, and bone marrow was flushed out with DMEM/Ham's F-12 hybrid medium containing 10% FBS. The pooled marrow cells were dispersed by agitation in the syringe and plated in six-multitwell plastic culture plates (2 × 10^6^ cells/well). After 48 h, nonadherent cells were removed by replacing the medium and the adherent cells were cultured further for five days at 37 °C in a humidified 5% CO2, 95% air atmosphere with medium replacement every other day.

**RNA Extraction and Differential Display—**Total RNA was isolated from C3H10T1/2 cells and ATDC5 cells by a single-step method as described previously (6) and analyzed by differential display according to the manufacture's instruction (RNAmap, GenHunter, Nashville, TN). The DNA fragment of approximately 500-bp expressed only in undifferentiated ATDC5 cells was identified and subcloned into pCRII vector (Invitrogen, San Diego, CA), and its nucleotide sequence was determined.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF163095.

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A New Ha-ras Suppressor Gene

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determined with ALFred DNA Sequencer (Amsersham Pharmacia Bio-
tech, Uppsala, Sweden).

cDNA Library Construction and Isolation of A-C1 cDNA—Oligo(dT)-
primed cDNA library from undifferentiated ATDC5 poly(A)− RNA was
constructed in λZAP Express vector (Stratagene, La Jolla, CA), and 1 × 106
plaque transformants were analyzed. The 500-bp cDNA fragment was
isolated from a 10% agarose gel electrophoresis, and transferred on Nytran
membranes (Schleicher & Schuell, Dassel, Germany). A 3.0-kb cDNA frag-
ment of A-C1 was used for hybridization as a probe. In analysis of tissue
expression, the membranes were exposed to X-Omat films (Eastman
Kodak, Rochester, NY) at −80 °C with Cronex lightning plus intensifying
screens (DuPont).

RT-PCR—The RT-PCR was performed as described previously (6).
Briefly, first-strand cDNA was synthesized using SuperScript II RNase
H-negative transcriptase (Life Technologies, Inc.) with 5 µg of total RNA
derived from various cell lines cultured in vitro. The following specific
primers were used: 5′-CACACTGGTAAAGGGCAAGCAGCC-3′ (sense primer)
and 5′-GGATGGTGCTCTTGAGCCCTGG-3′ (antisense primer) for mouse A-C1 cDNA. Amplification consisted of initial
denaturation at 94 °C for 5 min, followed by 25 reaction cycles (30 s at 94 °C,
30 s at 60 °C, and 30 s at 72 °C). Aliquots (8 µl) of each PCR
products were resolved on 3% NuSieve 3:1 agarose gels (FMC BioProd-
tect, Uppsala, Sweden).

RESULTS

Cloning and the Structure of A-C1 cDNA—To find genes specifically expressed in chondrogenic cells, we performed dif-
erential display using the total RNA extracted from a chondrogenic EC cell line, ATDC5, and obtained a 500-bp cDNA
fragment corresponding to the 3′-untranslated sequence of a novel differentially expressed gene named A-C1. This cDNA
fragment was then used as a probe to screen at high stringency a mouse cDNA library generated from ATDC5 cells. Ten cDNA
clones were obtained from 1 × 106 independent plaques. Eight out of ten clones contained a cDNA of about 3.0 kb. Fig. 1A
shows the nucleotide sequence of A-C1 cDNA and the deduced amino acid sequence of the putative A-C1 protein. A-C1 cDNA
contains a 501-bp open reading frame starting from an ATG
and an isoelectric point of 6.1. The 3′-end of the sequence contains a poly(A) stretch, preceded by a putative polyadenyl-
ation signal (AATAAA). A hydrophobicity plot using the Kyte-
Doolittle algorithms (23) showed the existence of a possible

2 M. Noda and H. Kitayama, unpublished data.
transmembrane domain at its C terminus (143–159 amino acid) (Fig. 1B). The amino acid sequence lacks the N-terminal signal peptide. These data suggest that the A-C1 cDNA is likely to encode an intracellular, membrane-bound protein. The amino acid sequence of A-C1 protein showed a significant homology with that of rat Ha-rev107 (4) (46% amino acid identity at the amino acid level) (Fig. 2).

Expression of A-C1 Protein by in Vitro Transcription/Translation—To confirm that the cloned 3.0-kb A-C1 cDNA contained a functional open reading frame, we performed an in vitro transcription/translation using the rabbit reticulocyte lysate system in the presence of [35S]methionine. A single protein product of the expected size was detectable. (Fig. 3). No protein product was detectable with the vector control (pcDNA3.1) (data not shown).

Transient Expression and Subcellular Localization of A-C1 Protein—To analyze the subcellular localization of A-C1 protein (Fig. 4), COS-7 cells were transiently transfected with a pcDNA3.1 plasmid containing the A-C1 cDNA with a FLAG tag sequence at the C terminus (pCMV/A-C1FLAG). Immunocytochemical staining with monoclonal anti-FLAG M2 antibody showed that A-C1 protein was localized in the cytoplasm and the perinuclear region but not within the nucleus. No staining was seen after transient transfection of the vacant pcDNA3.1 vector.

Expression of A-C1 mRNA in Various Cell Lines—We assessed by Northern analysis and RT-PCR the expression of A-C1 mRNA in culture cell lines: ATDC5, C3H10T1/2, MC3T3-E1, NIH3T3, Balb/c 3T3, ST2, and C2C12. A major hybridization band of about 3.2 kb was detected in chondrogenic ATDC5 cells, but not in C3H10T1/2 cells (Fig. 5A). The expression of A-C1 mRNA was detectable by RT-PCR in C2C12 as well as ATDC5, but not in MC3T3-E1, NIH3T3, Balb/c 3T3, and ST2 (Fig. 5B).

Expression of A-C1 mRNA in the Adult Mice Tissues—Northern analysis showed that among the various adult mice tissues, A-C1 mRNA was expressed highly in skeletal muscle and moderately in heart and brain (Fig. 6A). The expression of A-C1 mRNA was detectable also in bone marrow cells by RT-PCR (Fig. 6B). By in situ hybridization, A-C1 mRNA was localized in hippocampus and bone marrow in adult mice (Fig. 7).

Biological Activity of A-C1—The deduced amino acid sequence of A-C1 showed homology with that of rat Ha-rev107, suggesting that A-C1 may possess the revertant-inducing activity on ras-transformed NIH3T3 cells. We assessed such activity by transfecting A-C1 gene into NIH3T3 cell line transformed by Ras1V12 or v-Ki-ras oncogene (Lras/NIH and DT, respectively) and observing the morphology. When Lras/NIH cells were transfected with pCMV-A1, the colonies of these transfectants, observed under a phase-contrast microscope, were relatively smaller in size than those in the control culture transfected with pcDNA3.1 vector. Moreover, some of these product was detectable with the vector control (pcDNA3.1) (data not shown).

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colonies consisted of flat cells with increased attachment to the substrate (Fig. 8). The frequency of flat colonies was comparable with that observed after transfection of the control Krev-1 gene (Table I). Total numbers of colonies, however, were similar between the vector-transfected and pCMV-AC1-transfected cultures. In contrast, pCMV-AC1 did not give rise to flat colonies when transfected into DT cells (Fig. 8), while Krev-1 showed a substantial activity to induce flat colonies in this cell line (Table I).

Overexpression of A-C1 suppressed not only the transformed morphology of Lras/NIH cells but also the growth of these cells (Fig. 9). Doubling times of the pooled A-C1-transfected and vector-transfected Lras/NIH cells were \( \frac{20}{34} \) h, respectively. Saturation densities, however, were similar between the two \( \left( \frac{2.2 \times 10^5}{2.2 \times 10^5} \right) \) cells/cm².

**FIG. 4.** Subcellular localization of A-C1 protein. pcDNA3.1 vector or pcDNA3.1 vector as a control, was transiently transfected into COS-7 cells, and the FLAG-tagged A-C1 protein was detected using monoclonal anti-FLAG M2 antibody. Original magnification, × 100. Three independent experiments were performed and gave similar results.

**FIG. 5.** Expression of A-C1 mRNA in various cell lines. *A*, 5 μg/lane of poly(A)⁺ RNA was analyzed by Northern blot hybridization using 3.0-kb A-C1 cDNA (top) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (bottom). *B*, total RNA was analyzed by RT-PCR. Aliquots (8 μl) of the PCR products were resolved on 3% agarose gels. Three independent experiments were performed and gave similar results.

**FIG. 6.** Expression of A-C1 mRNA in various adult mouse tissues. *A*, multiple tissue blot containing 2 μg of poly(A)⁺ RNA from various mouse tissues (CLONTECH) was hybridized with the A-C1 cDNA (top) and a β-actin probe (bottom). The mRNA in each lane was isolated from heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and testis (lane 8). A DNA fragment of β-actin was also hybridized to the same blots as a control. *B*, total RNA was isolated from the cultured adherent bone marrow cells. RT-PCR was performed, and aliquots (8 μl) of the PCR product were resolved on 3% agarose gels. Three independent experiments were performed and gave similar results.

(Fig. 9). Doubling times of the pooled A-C1-transfected and vector-transfected Lras/NIH cells were −20 and −34 h, respectively. Saturation densities, however, were similar between the two \( \left( −2.2 \times 10^5 \right) \) cells/cm².

**DISCUSSION**

We have isolated by differential display a novel gene, A-C1, encoding a protein of 167 amino acids, which is specifically expressed in skeletal muscles, heart, brain, and bone marrow.
in vivo. A-C1 protein has a putative transmembrane domain at the C terminus (143–159 amino acid) and lacks the N-terminal signal peptide (Fig. 1), indicating that A-C1 protein is probably an intracellular, membrane-bound protein. Indeed, a preliminary experiment with tagged protein described here suggested its predominant localization in the cytoplasm and perinuclear region (Fig. 4). Further studies are required, however, to confirm the subcellular localization and membrane-association of the intact, endogenous A-C1 protein under physiological conditions.

The amino acid sequence of A-C1 showed 46% homology with that of rat Ha-rev107 (4). Ha-rev107 is a class II tumor suppressor, as defined by its down-regulation after Ha-ras transformation in fibroblasts, expression in ras-resistant fibroblasts, and growth-inhibiting capacity in Ha-ras-transformed cell lines. The expression of Ha-rev107 was detected in liver, kidney, stomach, and intestine, distinct from that of A-C1, while Ha-rev107 protein appeared to be linked to the nuclear membrane and to membranes in the perinuclear space, similar to the subcellular localization of A-C1. Ras proteins bind guanine...
nucleotides with high affinity. Three sequence motifs important for nucleotide interaction have been determined, which are conserved between different guanosine nucleotide-binding proteins (12): GXXGXS is involved in the binding to the α- and β-phosphates; DXG is involved in binding to Mg²⁺ and γ-phosphate when GTP is bound; and NKXD is important for binding to the guanine ring. Both A-C1 and Ha-rev107 protein have two consensus sequence motifs, DXG and NKXD (Fig. 2), which were previously unnoticed. The role of these motifs should be elucidated in future studies. Overexpression of A-C1 not only induced reversion of morphology in Ha-ras-transformed NIH3T3 cells but also suppressed the growth of these cells, as evidenced by the smaller colony formed (Fig. 8) and the growth curve (Fig. 9). These results support the notion that A-C1 may serve as a negative regulator for the Ha-ras-mediated signaling pathway. How this is achieved is also an important subject for future studies. Our data also indicated that A-C1 is not an effective suppressor against the v-Ki-ras-transformed cell line we used. Whether this reflects some kind of selectivity among Ras family proteins is an interesting question to be clarified.

In this study, we identified the A-C1 gene by differential display to screen genes specifically expressed in mouse chondrogenic EC cells, ATDC5. Our observations that A-C1 is expressed in specific tissues in vivo and certain cell lines in vitro and that A-C1 is a potential inhibitor of Ha-ras-mediated intracellular signaling pathway raise the possibility that A-C1 may play a role in the regulation of cell growth and differentiation in these specific tissues.