Aortic Carboxypeptidase-like Protein, a Novel Protein with Discoidin and Carboxypeptidase-like Domains, Is Up-regulated during Vascular Smooth Muscle Cell Differentiation*

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Phenotypic modulation of vascular smooth muscle cells plays an important role in the pathogenesis of arteriosclerosis. In a screen of proteins expressed in human aortic smooth muscle cells, we identified a novel gene product designated aortic carboxypeptidase-like protein (ACLP). The ~4-kilobase human cDNA and its mouse homologue encode 1158 and 1128 amino acid proteins, respectively, that are 85% identical. ACLP is a nonnuclear protein that contains a signal peptide, a lysine- and proline-rich 11-amino acid repeating motif, a discoidin-like domain, and a C-terminal domain with 39% identity to carboxypeptidase E. By Western blot analysis and in situ hybridization, we detected abundant ACLP expression in the adult aorta. ACLP was expressed predominantly in the smooth muscle cells of the adult mouse aorta but not in the adventitia or in several other tissues. In cultured mouse aortic smooth muscle cells, ACLP mRNA and protein were up-regulated 2–3-fold after serum starvation. Using a recently developed neural crest cell to smooth muscle cell in vitro differentiation system, we found that ACLP mRNA and protein were not expressed in neural crest cells but were up-regulated dramatically with the differentiation of these cells. These results indicate that ACLP may play a role in differentiated vascular smooth muscle cells.

Vascular smooth muscle cells (VSMCs)1 are the predominant component of the blood vessel wall, where their principal function is to regulate vascular tone (1). Although VSMCs normally exist in a differentiated state, they can dedifferentiate and proliferate in response to certain stimuli. Activation of VSMCs from a contractile and quiescent state to a proliferative and synthetic state contributes to several disease processes, including arteriosclerosis (2). Defining effectors that modulate VSMC function and identifying marker proteins that characterize a given VSMC phenotypic state will contribute to our understanding of the mechanisms regulating VSMC differentiation (1).

The origins of VSMCs during embryonic development are diverse (reviewed in Refs. 1, 3, and 4). During development, VSMCs derive from many cell types, such as local mesodermal precursors and neural crest cells (3, 5). Despite the fact that they express a similar set of smooth muscle cell marker genes, these cell populations can differ in morphology and respond in a lineage-dependent manner to factors such as transforming growth factor-β1 (6). An understanding of the complex regulation of smooth muscle cell differentiation requires the identification of proteins involved in this response.

In a search for potential markers and regulators of smooth muscle cell growth and differentiation, we identified a novel gene product termed aortic carboxypeptidase-like protein (ACLP). ACLP contains a signal peptide, a repeating motif, a discoidin-like domain, and a domain with homology to the carboxypeptidases. ACLP is expressed highly in adult aortic smooth muscle cells, as detected by Northern blotting, Western blotting, and in situ hybridization. Also, expression of ACLP increases in cultured aortic smooth muscle cells after serum starvation. Using a recently developed in vitro system that allows the differentiation of multipotential mouse neural crest cells into smooth muscle cells, we show that ACLP is up-regulated dramatically. These results suggest that ACLP may play a role during development in the acquisition by VSMCs of the differentiated phenotype.

**EXPERIMENTAL PROCEDURES**

Cell Lines, Cell Culture, and Reagents—Rat aortic smooth muscle cells (RASMCs) and mouse aortic smooth muscle cells (MAMSCs) were isolated by the method of Gunther et al. (7) from the thoracic aortas of adult male Sprague-Dawley rats and C57Bl/6 mice. Human aortic smooth muscle cells (HASMCs) were purchased from Clonetics (San Diego, CA), and rat A7r5 smooth muscle cells and C2C12 mouse myoblasts were purchased from the ATCC (Rockville, MD). The mouse neural crest cell line Monc-1 was provided by David Anderson (Pasadena, CA). Monc-1 cells were cultured on fibronectin-coated plates as described (8), with minor modifications (9). RASMCs, MACs, and A7r5 cells were cultured in Dulbecco’s modified Eagle’s medium with 3.7 g/liter glucose (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 4 mM l-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 10 mM HEPES (pH 7.4). C2C12 cells were grown in Dulbecco’s modified Eagle’s medium supple-

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1 The abbreviations used are: VSMC, vascular smooth muscle cell; ACLP, aortic carboxypeptidase-like protein; RASMC, rat aortic smooth muscle cell; MASMC, mouse aortic smooth muscle cell; HASMC, human aortic smooth muscle cell; bp, base pair(s); kb, kilobase(s); AEBP1, adipocyte enhancer-binding protein 1; PCR, polymerase chain reaction.
mented with 15% fetal bovine serum, 4 mM t-glutamine, 100 µg/mL streptomycin, and 100 units/mL penicillin. HASMCs were cultured in M199 medium (Life Technologies, Inc.) supplemented with 20% fetal bovine serum, 4 mM t-glutamine, 100 µg/mL streptomycin, and 100 units/mL penicillin. Cells were grown at 37 °C in a humidified incubator containing 5% CO2.

Cloning and Sequencing of Human and Mouse ACLP—A recombinant E47 fusion protein (N3-SH2[ALTA]) containing the basic helix loop helix domain of hamster shPan-1 (amino acids 509–646, with mutations R551A, V552L, and R553A) and a heart muscle kinase recognition sequence and FLAG epitope was expressed and purified as described (10, 11). The fusion protein was phosphorylated by immunopotent protein kinase in the presence of γ-32P[ATP] and then used to screen a human aorta χgt11 cDNA expression library (1.5 × 106 pfu; CLONTECH, Palo Alto, CA) by interaction cloning (10, 11). A 1450-base pair (bp) cDNA clone that resulted from this interaction cloning was radiolabeled by random priming and used to isolate an ~2.8-kilobase (kb) cDNA clone from the same human aorta χgt11 cDNA library. Because Northern blotting revealed that the latter was also a partial cDNA clone, we isolated additional 5′ sequences from HASMC RNA by 5′ rapid amplification of cDNA ends (Life Technologies, Inc.).

GenBankTM searches revealed significant homology between the 3′ end of our human ACLP clone and mouse adipocyte enhancer-binding protein 1 (AEBP1) (12). To isolate the corresponding mouse cDNA, we PCR amplified fragments of the first-stranded reverse transcriptase-cDNA and total RNA by reverse transcription with the primer 5′-ATCTGGTTTTGCTCAAT-3′, which was designed according to the 5′ end of mouse AEBP1 (12). Using the nested primer 5′-GTAGCTCACCTCCGATAG-3′ and the anchor primer included in the kit for 5′ rapid amplification of cDNA ends, we amplified an ~1400-bp fragment by the polymerase chain reaction (PCR). This product was ligated into pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced as described below. The entire open reading frame of mouse ACLP was then amplified from C2C12 RNA by reverse transcription PCR (Expand Long Template PCR System, Boehringer Mannheim) and ligated into pCR2.1. We sequenced the human and the mouse clones by the dideoxy nucleotide chain termination method, using a combination of Sequenase Version 2.0 (Amersham Pharmacia Biotech), the Thermo Sequenase33P terminator cycle sequencing kit (Amersham Pharmacia Biotech), and the Thermo Sequenase fluorescent-labeled cycle sequencing kit with 7-deaza-GTP (Schleicher and Schuell) in 48× mTris, pH 8.3, 39 mM glycine, 0.037% sodium dodecyl sulfate, and 20% methanol transfer buffer. Blots were equilibrated with 25× mTris, pH 8, 125 mM NaCl, and 0.1% Tween 2 and blocked in the same solution containing 4% nonfat dry milk. Blots were incubated with anti-ACLP serum diluted 1:1000 and then with horseradish peroxidase-conjugated goat anti-rabbit serum diluted 1:4000. Membranes were processed with an enhanced chemiluminescence reagent (NEN Life Science Products) and exposed to film.

In Situ Hybridization—Adult male Sprague-Dawley rats were perfused with 4% paraformaldehyde, and their organs were removed and sectioned (19). Probe was prepared, and in situ hybridization was conducted as described (19, 20). ACLP mRNA was detected with a 32P-labeled antisense riboprobe synthesized with SP6 RNA polymerase from a linearized 0.7-kb fragment of ACLP cDNA in pCR2.1. As a control, a sense RNA probe was synthesized with T7 RNA polymerase from a linearized ACLP cDNA fragment in pCR2.1.

RESULTS

Isolation and Characterization of Human and Mouse ACLP cDNAs—To identify proteins interacting with products of the E2A gene (E12/E47) in VSMCs, we screened a human aorta expression library with a 32P-labeled E47 fusion protein. One truncated clone isolated from this screen (number 11) led to the full-length ACLP clone characterized here. Using in vitro binding assays, we determined that proteins derived from clone 11, but not from the full-length protein, bound to E12 and E47 (data not shown). The 3935 bp, full-length human ACLP cDNA contains an open reading frame of 1158 amino acids (Fig. 1A) and a Kozak consensus sequence for initiation of translation (GCCATGG) (21) preceded by an in-frame stop codon. The protein has a calculated molecular mass of 130 kDa and an estimated pI of 4.8, and it contains a putative signal peptide sequence (22, 23), an 11 amino acid lysine- and proline-rich motif repeated four times at the N terminus, a domain with 30% amino acid identity to the slime mold adhesion protein discoidin I, and a C-terminal domain with 39% identity to carboxypeptidase E (Fig. 1B).

GenBankTM searches revealed that the C terminus of human ACLP is highly homologous to mouse AEBP1 (12). AEBP1 was originally identified as a ~2.5-kb cDNA that hybridized to an 4-kb band on Northern blot analysis, and it is predicted to encode 719 amino acids, 79 kDa protein. The homology between ACLP and AEBP1 suggested two possibilities: either ACLP was a longer member of the AEBP1 gene family, or the AEBP1 sequence was substantially truncated at its 5′-end. To test the two possibilities, we cloned the mouse homologue of ACLP by a combination of 5′ rapid amplification of cDNA ends and reverse transcription PCR. After sequencing the 3633-bp...
mouse ACLP cDNA fragment, we found that it encoded an open reading frame (1128 amino acids) similar to that of our human clone, indicating that it is the mouse homologue (the two are 85% identical and 90% similar). Because AEBP1 is identical to the C terminus of the mouse ACLP, we conclude that the AEBP1 cDNA is probably not complete (the start of the AEBP1 sequence is indicated by a bullet in Fig. 1A).

Characterization of ACLP—To confirm the putative open reading frame of mouse ACLP, we performed in vitro transcription and translation reactions with the mouse cDNA used as template. Translated products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and a prominent band of 175 kDa was detected (Fig. 2A). To identify the endogenous ACLP, a C-terminal fragment of mouse ACLP was

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**Fig. 1. Deduced amino acid sequences of human and mouse ACLP.** A, deduced open reading frames of human ACLP and mouse ACLP. The human and mouse proteins contain 1158 and 1128 amino acids, respectively. Bullet marks initiating methionine in mouse AEBP1. Highlighted motifs include a signal peptide (boldface, underlined), a 4-fold lysine- and proline-rich repeating motif (boldface italic), a discoidin-like domain (boldface italic, underlined), and a region with homology to the carboxypeptidases (boldface).

B, schematic representation of human ACLP. Marked are the signal peptide sequence at the N terminus (Signal), the 4-fold repeating motif (Repeat), the discoidin-like domain (DLD), and the region with homology to the carboxypeptidases (CLD).
expressed in bacteria, purified, and used to raise antibodies in rabbits. By Western blot analysis, this antibody detected a single band with an apparent mobility of ~175 kDa in MASMC extracts (Fig. 2B). The similar migration of the endogenous ACLP and the protein transcribed and translated in vitro indicates that we isolated a full-length cDNA clone.

To assess the subcellular localization of ACLP, we generated a mouse ACLP expression construct with a c-myc epitope at the C terminus. The myc epitope was placed at the C terminus so that it would not interfere with signal peptide-mediated processes. This construct was transfected transiently into RASMCs and A7r5 cells, and immunostaining was performed with anti-c-myc antibody 9E10. RASMCs and A7r5 cells (Fig. 3, A and C) both exhibited strong membrane-associated or cytoplasmic staining. Staining was most intense in the perinuclear region and was not observed in the nucleus (Fig. 3, B and D).

Tissue Expression of Mouse ACLP—Although the ACLP cDNA was cloned originally from aortic smooth muscle cells, we also wanted to examine its mRNA and protein expression in other tissues. As expected, levels of ACLP mRNA were high in the whole aorta (including adventitia) (Fig. 4A). Also, ACLP message was present in other tissues, including the colon and the kidney (Fig. 4A). To examine expression of ACLP, we subjected extracts from mouse tissues to Western blot analysis. ACLP was expressed abundantly in the mouse aorta (without adventitia) but not in the adventitia, heart, liver, skeletal muscle, or kidney (Fig. 4B). The presence of ACLP mRNA in the kidney (Fig. 4A) but absence of protein may indicate translational regulation. To identify cell types expressing ACLP in the adult, we performed in situ hybridization on adult rat aorta and skeletal muscle. The antisense riboprobe detected specific ACLP expression in the smooth muscle cells of the aorta (Fig. 5A), whereas the control, sense probe did not (Fig. 5B). As expected, neither the sense nor the antisense probe hybridized to skeletal muscle cells (Fig. 5, C and D).

ACLP Expression in Cultured Smooth Muscle Cells—Because ACLP expression was high in the differentiated smooth muscle cells of the aorta (Fig. 3B), we examined the effect of

![Fig. 2. In vitro transcription and translation of mouse ACLP and identification of ACLP. A, the mouse ACLP cDNA in pCR2.1 was transcribed and translated in vitro in the presence of [35S]methionine. An aliquot of this reaction was resolved on a 6% sodium dodecyl sulfate-polyacrylamide gel that was then dried and exposed to film at room temperature. B, Western blot analysis of proteins extracted from MASMCs. After total cellular protein lysates had been prepared as described under “Experimental Procedures,” 50-μg aliquots were resolved on a 6% sodium dodecyl sulfate-polyacrylamide gel. The gel was transferred to a nitrocellulose membrane and incubated with a polyclonal anti-ACLP primary antiserum and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. The blot was visualized by enhanced chemiluminescence and exposed to film at room temperature.](image)

![Fig. 3. Cellular localization of mouse ACLP. RASMCs and A7r5 cells were transfected transiently with an ACLP expression construct tagged with c-myc at the C terminus. Fusion protein was detected with an anti-c-myc antibody (A and C), and nuclear DNA was counterstained with Hoechst 33258 (B and D). RASMCs and A7r5 cells both exhibited strong perinuclear staining that was excluded from the nucleus, as demonstrated by nuclear DNA counterstaining. Initial magnification, × 400.](image)

![Fig. 4. ACLP mRNA and protein expression in mouse tissue. A, total RNA was isolated from mouse tissues as described under “Experimental Procedures,” and 10-μg aliquots were resolved on an agarose-formaldehyde gel, transferred to a nitrocellulose filter, and hybridized to a 32P-labeled fragment of mouse ACLP. Equal loading was verified by hybridization to a 32P-labeled oligonucleotide complementary to the 18S ribosomal RNA. B, total cellular protein was extracted from mouse organs, and 50-μg aliquots were subjected to Western blotting with a polyclonal anti-ACLP antiserum, which was detected with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.](image)
hybridized with [35S]UTP-labeled antisense (A and C) or sense (B and D) riboprobes. Magnification, × 600.

FIG. 5. Detection of ACLP mRNA in aorta by in situ hybridization. Rats were perfused with 4% paraformaldehyde, and tissue was removed and sectioned as described under “Experimental Procedures.” Sections from aorta (A and B) and skeletal muscle (C and D) were hybridized with [35S]UTP-labeled antisense (A and C) or sense (B and D) riboprobes. Magnification, × 600.

FIG. 6. Increase in ACLP mRNA and protein levels in serum-starved aortic smooth muscle cells. A, total cellular RNA was extracted from MASMCs and RASMCs cultured in 10% fetal bovine serum (Growing) or 0.4% calf serum (Quiescent) for 3 days. RNA was fractionated on a 1.2% agarose-formaldehyde gel, which was transferred to a nitrocellulose filter, hybridized to a 32P-labeled fragment of mouse ACLP, and normalized to 18S rRNA. B, MASMCs treated as in A were harvested for total cellular protein and immunoblotted as described in the legend to Fig. 2B.

sage and protein levels are modest, they are consistent with increases in VSMC differentiation-specific markers observed in other systems (24, 25).

ACLP Expression in Smooth Muscle Cell Differentiation—Our laboratory recently developed an in vitro system for differentiating smooth muscle cells from Monc-1 cells, a mouse line derived from the neural crest (9). Monc-1 cells differentiate into smooth muscle cells when medium supplemented with chick embryo extract is replaced with differentiation medium (9). To examine ACLP expression during the conversion of undifferentiated Monc-1 cells to smooth muscle, we measured the time course of ACLP expression. ACLP mRNA was nearly undetectable in undifferentiated Monc-1 cells (Fig. 7A). As the cells differentiated, however, ACLP expression increased until it became marked at days 4 and 6 after the start of differentiation (Fig. 7A). Under these conditions, induction of ACLP appeared to lag behind that of smooth muscle α-actin, a marker for smooth muscle cells. To compare the level of ACLP in cells treated similarly, we prepared protein extracts from undifferentiated Monc-1 cells and cells allowed to differentiate for 6 days (Fig. 7B). ACLP was not detectable in undifferentiated Monc-1 cells (day 0) but was expressed highly (day 6) under conditions that promote Monc-1 cell differentiation into smooth muscle cells. The abundance of ACLP in these cells was similar to that in MASMCs.

DISCUSSION

We have cloned a novel cDNA from human aortic smooth muscle cells, termed ACLP, and its mouse homologue. Notable features of the protein include a predicted signal peptide sequence at the N terminus, a lysine- and proline-rich 11-amino acid repeat, a discoidin-like domain, and a large C-terminal carboxypeptidase-like domain (Fig. 1).

The screen that led to the identification of ACLP was performed to identify binding partners of the E2A proteins. The products of the E2A gene, E12 and E47, serve as heterodimerization partners for tissue specific transcription factors that regulate growth and differentiation in several cell types. Although the E2A gene products are expressed ubiquitously (26), a vascular smooth muscle specific heterodimerization partner or transcription factor has not been identified. We cloned the C-terminal portion of human ACLP (amino acids 793–1158) by using a labeled E47 protein probe and verified its binding to E47 by in vitro assays (data not shown). However, the full-length ACLP, because of its predicted signal peptide sequence (Fig. 1) and nonnuclear subcellular localization (Fig. 3), probably does not function as a heterodimerization partner for E47 in vivo.

GenBank™ searches indicated high homology between the C terminus of human ACLP and the mouse AEBP1 described by He et al. (12). To determine the relation between ACLP and
AEBP1, we cloned the mouse ACLP cDNA. By sequence comparison, AEBP1 was found to be identical to mouse ACLP, beginning at ACLP methionine 410 (Fig. 1A). We then determined that ACLP is a single-copy gene in the mouse and cloned the region corresponding to the 5’ end of AEBP1 from genomic DNA.2 Analysis of the genomic clone confirmed that the AEBP1 sequence is missing a G residue 11 bases 5’ to the identified ATG. The presence of this G residue in ACLP would eliminate the in frame stop codon proposed by He et al. (12) and extend the open reading frame.

The 2.5-kb AEBP1 cDNA is unlikely to code for an authentic protein. Probes derived from AEBP1 and both the 5’ and 3’ ends of ACLP detected a single, ~4-kb band by Northern blot analysis, which is consistent with the size of the human as well as the mouse ACLP cloned cDNAs. Because the AEBP1 cDNA contains a putative polyadenylation signal and a poly(A) tail, the difference between the AEBP1 cDNA and mRNA is ~1.5 kb. This missing 1.5 kb of sequence is present in the 5’ end of the ACLP cDNA. Also, the anti-ACLP antibody generated for these studies was raised from the C terminus of ACLP, which is identical to AEBP1. The antibody detected only a single band of ~175 kDa by Western blotting in several tissues examined (Fig. 4B), which is consistent with the mobility of ACLP transcribed and translated in vitro (Fig. 2). We also detected a single band of identical mobility in protein extracts from several cell lines in culture, including 3T3-L1 preadipocytes. ACLP was expressed in 3T3-L1 preadipocytes at substantially lower levels than in MASMCs or differentiated Monc-1 cells (data not shown). Thus, AEBP1 appears to be a truncated clone of mouse ACLP. AEBP1 is missing the ACLP signal peptide, repeat domain, and part of the discoidin domain.

ACLP has a prominent carboxypeptidase-like domain of about 500 amino acids at its C terminus (Fig. 1B). This domain is 39% identical to carboxypeptidase E. Despite this high sequence similarity, however, we and others (27) have been unable to demonstrate that this domain of ACLP has any catalytic carboxypeptidase activity. These results may reflect the divergence of specific residues in ACLP from sequences of the carboxypeptidase family (27). For example, a histidine involved in zinc binding in carboxypeptidases is replaced by an asparagine (amino acid 763) in human ACLP. Catalytically important tyrosine and glutamic acid residues in the carboxypeptidases are substituted by asparagine (amino acid 852) in human ACLP. Catalytically involved in zinc binding in carboxypeptidases is replaced by an asparagine (amino acid 700) in human ACLP. AEBP1 appears to be a truncated clone of mouse ACLP. AEBP1 is missing the ACLP signal peptide, repeat domain, and part of the discoidin domain.

ACLP and Smooth Muscle Cell Differentiation

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