Molecular Cloning and Characterization of SmLIM, a Developmentally Regulated LIM Protein Preferentially Expressed in Aortic Smooth Muscle Cells*

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Mukesh K. Jain, Kenji P. Fujita, Chung-Ming Hsieh, Wilson O. Endege, Nicholas E. S. Sibinga‡, Shaw-Fang Yet, Saori Kashiki, Wen-Sen Lee‡, Mark A. Perrella‡, Edgar Haber‡, and Mu-En Lee§

From the Cardiovascular Biology Laboratory, Harvard School of Public Health, the Department of Medicine, Harvard Medical School, and the Cardiovascular and Pulmonary Divisions, Brigham and Women's Hospital, Boston, Massachusetts 02115

Differentially, quiescent vascular smooth muscle cells assume a dedifferentiated, proliferative phenotype in response to injury, one of the hallmarks of arteriosclerosis. Members of the LIM family of zinc-finger proteins are important in the differentiation of various cells including striated muscle. We describe here the molecular cloning and characterization of a developmentally regulated smooth muscle LIM protein, SmLIM, that is expressed preferentially in the rat aorta. This 194-amino acid protein has two LIM domains, and comparisons of rat SmLIM with its mouse and human homologues reveal high levels of amino acid sequence conservation (100 and 99%, respectively). SmLIM is a nuclear protein and maps to human chromosome 3. SmLIM mRNA expression was high in aorta but not in striated muscle and low in other smooth muscle tissues such as intestine and uterus. In contrast with arterial tissue, SmLIM mRNA was barely detectable in venous tissue. The presence of SmLIM expression within aortic smooth muscle cells was confirmed by in situ hybridization. In vitro, SmLIM mRNA levels decreased by 80% in response to platelet-derived growth factor-BB in rat aortic smooth muscle cells. In vivo, SmLIM mRNA decreased by 60% in response to vessel wall injury during periods of maximal smooth muscle cell proliferation. The down-regulation of SmLIM by phenotypic change in vascular smooth muscle cells suggests that it may be involved in their growth and differentiation.

In their normal state, vascular smooth muscle cells (VSMCs) regulate vessel tone and blood pressure. VSMCs are not terminally differentiated, in contrast with skeletal muscle and cardiac muscle cells. In response to mechanical, chemical, or immunologic injury (1–5) the VSMC phenotype changes rapidly from that of a differentiated, quiescent cell to that of a dedifferentiated, proliferating cell. Although VSMC proliferation is a hallmark of arteriosclerosis, the leading cause of death in developed countries, little is known about the molecular mechanisms regulating this phenotypic change. Progress in this area has been limited by the lack of VSMC-specific markers and precursor cells that can be differentiated into VSMCs in vitro (6).

Unlike VSMC differentiation, skeletal muscle differentiation is well studied. The myogenic helix-loop-helix proteins MyoD, myogenin, myf-5, and myf-6 have been assigned important roles in the differentiation of skeletal muscle cells (7–10). Recently, a muscle LIM-domain protein, MLP, has also been described as a positive regulator of myogenic cell differentiation (11). Its cysteine-rich LIM domain, defined by the 50–60-amino acid consensus sequence (CX$_2$-CX$_{17}$ ± 1-H-X$_2$-C)-X$_2$-(C-X$_2$-C)-X$_{17}$ ± 1-C-X$_2$-D/H) (12), is found in proteins that function in developmental regulation, cellular differentiation, and actin-based cytoskeletal interaction (13–15). Because this sequence is conserved among highly divergent species, the LIM domain appears to be functionally important (16).

So far there are three classes of LIM proteins. Class 1 proteins (LIM-HD) contain two LIM domains and a homeodomain. Lin-11, Isl-1, and Mec-3 (17–19), the first LIM proteins to be identified, belong to this group. Class 2 proteins (LIM-only) contain one or more LIM domains, but lack the homeodomain (14). Class 3 proteins (LIM-K) contain LIM domains and a protein kinase domain (20–22).

Two members of the LIM-only class, RBTN2 (12) and MLP (11), have been shown to play important roles in cellular differentiation. Originally identified in childhood T cell acute lymphoblastic leukemia (23, 24), RBTN2 is essential for erythroid cell development; a homozygous null mutation in RBTN2 leads to failure of yolk sac erythropoiesis and embryonic death (12). MLP is expressed only in the heart and skeletal muscle of rats. Overexpression of sense MLP in C2 myoblasts potentiates myogenic cell differentiation. In contrast, expression of antisense MLP retards myoblast differentiation and withdrawal from the cell cycle. Although these observations suggest that MLP could be involved in regulating skeletal and heart muscle cell-specific gene expression (11), MLP mRNA is not expressed in VSMCs in vitro or in vivo (data not shown). We hypothesized that a related but heretofore unidentified LIM protein may play an analogous role in VSMC differentiation.

We report the identification and characterization of a LIM-
only protein expressed preferentially in aortic smooth muscle cells. Smooth muscle LIM (SmLIM) is a nuclear protein whose expression is regulated developmentally. Stimulation of cultured VSMCs with the potent mitogen platelet-derived growth factor (PDGF)-BB caused a down-regulation of SmLIM mRNA. In vivo, SmLIM mRNA levels decreased as VSMCs changed from a quiescent to a proliferative phenotype in response to vascular injury.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Aortic smooth muscle cells were harvested from the thoracic aorta of adult male Sprague-Dawley rats (200–250 g) by enzymatic digestion according to the method of Gunther et al. (25). COS-7 and 10T1/2 cells were obtained from the American Type Cell Culture Collection. Embryonic stem cells (D3) were kindly provided by R. Rosenberg (Massachusetts Institute of Technology, Boston, MA). Rat aortic smooth muscle cells were grown in DME (I RH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 μm Hepes (pH 7.4) in a humidified incubator (37 °C, 5% CO2). COS-7 and 10T1/2 cells were grown similarly, with the exceptions that DME was supplemented with Serum Plus (HyClone, Logan, Utah) for the former and basal medium Eagle (I RH Biosciences) was substituted for DME for the latter. Cells were cultured and maintained in an undifferentiated state with leukemia inhibitory factor as described by Doetschman et al. (26). PDGF-BB was purchased from Collaborative Biomedical Products (Bedford, MA).

Cloning and Sequencing of Rat (r)-SmLIM and Human (h)-SmLIM—The full-length MLP cDNA was amplified from rat heart RNA by the reverse transcriptase PCR (27). Forward (5′-GAGTCCTCACCAGTCGCAGAC-3′) and reverse (5′-CTCTCCACCCCCAAATTAG-3′) primers, designed according to the published rat MLP sequence (11), were used to amplify an 801-base pair fragment. The PCR fragment was then subcloned and sequenced by the DNA sequencing department (Promega) of this expression plasmid with wheat germ proteinase (Promega, Madison, WI) according to the manufacturer’s instructions. The subcloned and translated products were resolved on a 10% SDS-PAGE Tricine gel (23), and autoradiography was performed with Kodak BMR film at room temperature.

In Situ Hybridization—r-SmLIM mRNA was hybridized in situ as described elsewhere (33) with minor modifications. Adult male Sprague-Dawley rats were perfused with 4% paraformaldehyde. Organs were then postfixed with 4% paraformaldehyde, soaked in 30% sucrose until the tissue had sunk, embedded in optimum cutting temperature compound, and sectioned at 5 μm. Tissue sections were cut at a thickness of 5 μm. SmLIM mRNA was detected by hybridization with a 35S-UTP-labeled antisense cRNA probe synthesized with the SP6 RNA polymerase from HindIII-linearized r-SmLIM in Bluescript II SK+ (Stratagene, La Jolla, CA). For control experiments, a 35S-UTP-labeled sense cRNA probe was synthesized under the same conditions. RNA probes were degraded to a length of approximately 100–200 nucleotides by partial hydrolsis for 15 min at 60 °C in 80 μg/ml NaHCO3 and 200 μg/ml Na2CO3. After hybridization the tissue sections were washed under moderately stringent conditions as previously described (33). The dried tissue sections were then dipped into Kodak NTB2 emulsion (Eastman Kodak Co.) and exposed for 2–4 days at 4 °C. Counterstaining was performed with hematoxylin-eosin.

RESULTS

Isolation and Characterization of r-SmLIM and h-SmLIM cDNA—The nucleotide sequence of the r-SmLIM cDNA revealed a 582-base pair open reading frame encoding a 194-amino acid protein. Analysis of this frame identified two LIM domains separated by a glycine-rich region and a putative nuclear localization signal (Fig. 1A). The nucleotide sequence flanking the putative initiation methionine complied with the Kozak consensus sequence for initiation of translation (34), and the r-SmLIM open reading frame predicted a 21-kDa protein. We then cloned the entire r-SmLIM cDNA into the PCRIII eukaryotic expression vector. In vitro transcription and translation (Promega) of this expression plasmid with wheat germ lysate revealed a protein product of 21 kDa (Fig. 1B).

To determine whether SmLIM was conserved across species, we obtained the human (Fig. 2A) and mouse (m2) homologues. A comparison of the h-SmLIM and r-SmLIM open reading frames revealed 93% identity at the cDNA level and 99% identity at the amino acid level (Fig. 2, A and B). Comparison of the open reading frames of m-SmLIM and r-SmLIM revealed 97% identity at the cDNA level and 100% identity at the amino acid level (Fig. 2B). A GenBank search indicated that SmLIM shares homology with the cysteine-rich protein (CRP) family (13, 15, 35–37). Fig. 2A compares r-SmLIM and h-SmLIM with their rat and human CRP counterparts and rat MLP. Although an amino acid sequence comparison of r-SmLIM versus h-SmLIM shows 99% identity (Fig. 2B), a comparison of r-SmLIM with r-CRP shows 79% identity. These data indicate that SmLIM and CRP are related but different genes.

Cellular and Chromosomal Localization of SmLIM—The r-SmLIM deduced amino acid sequence contains the putative nuclear localization signal KKYGPK, suggesting that SmLIM

\[ ^{2}S-F.~Yet,~M.~K.~Jain,~and~M.-E.~Lee,~unpublished~observation. \]
may be a nuclear protein. To determine the cellular localization of SmLIM, we first generated a plasmid that would express a fusion protein of the c-Myc tag and SmLIM. This plasmid and the control vector alone were transfected into COS cells and immunostained with an anti-c-Myc antibody. Detection of the immunofluorescent signal in the nucleus of COS cells transfected with the c-Myc-r-SmLIM fusion plasmid but not the control vector alone localized the SmLIM protein to the nucleus (Fig. 3). We performed the same experiment in 10T1/2 fibroblasts and found that SmLIM localized to the nucleus in these cells as well (data not shown). We also mapped the chromosomal location of h-SmLIM with the BIOS somatic cell hybrid blot. h-SmLIM localized to chromosome 3 (Fig. 4).

Tissue Distribution of r-SmLIM and h-SmLIM (Northern Analysis)—Total RNA were isolated from 15 tissues of adult male and female rats and analyzed for SmLIM expression by Northern blot analysis (Fig. 5A). A single, intense, 1.0-kb band was detected in aorta. A much weaker signal was detected in kidney, thymus, and intestine. SmLIM expression was not detectable in heart and skeletal muscle and was barely detectable in brain, testis, esophagus, lung, liver, aortic adventitia, vena cava, and uterus. Thus, r-SmLIM appears to be expressed in tissue containing smooth rather than striated muscle. Furthermore, because expression of SmLIM was much greater in aorta than in intestine or uterus it would appear to be expressed preferentially in VSMCs. Even among vascular RNAs, r-SmLIM expression was more robust in arterial tissue (aorta) than in venous tissue (vena cava). Consistent with the r-SmLIM expression pattern, h-SmLIM was expressed to a high degree in aorta but not in heart or skeletal muscle (Fig. 5B).

Tissue Distribution of r-SmLIM (in situ Hybridization)—Fig. 6, left shows intense staining of r-SmLIM in aorta. A corresponding sense (control) experiment (Fig. 6, right) was performed to localize r-SmLIM expression within the vessel wall. Fig. 6, top left, shows intense staining of r-SmLIM in smooth muscle of the aortic wall.
h-SmLIM visible only in the transferred to nitrocellulose filters and hybridized with a32P-labeled respectively suppress among the smooth muscle cell mitogens in its ability to select-

Growth Factors and Arterial Wall Injury—PDGF-BB is unique in arterial smooth muscle cells.

analyses and indicate that r-SmLIM was expressed preferen-
magnitude reveals that r-SmLIM expression was limited to
both the aorta (Ao) and a small artery (Ar) nearby. Consistent with our Northern analysis, minimal expression of r-SmLIM was visible in the vena cava (V). A view of the aorta at higher magnification reveals that r-SmLIM expression was limited to smooth muscle cells in the medial layer (Fig. 6, bottom left). SmLIM signal expression was absent in skeletal muscle cells (data not shown). These observations agree with our Northern analyses and indicate that r-SmLIM was expressed preferentially in arterial smooth muscle cells.

Down-regulation of r-SmLIM Expression in VSMCs by PDGF-BB on SmLIM expression in cultured VSMCs. r-SmLIM mRNA levels decreased gradually in response to PDGF-BB stimulation (Fig. 7A). A decrease in r-SmLIM expression appeared as early as 4 h after treatment, and a maximal decrease of 80% was obtained at 32 h after treatment.

In response to vessel wall injury, VSMCs undergo a phenotypic change from a differentiated, contractile state to a dedifferentiated, proliferative state. Balloon injury of the rat carotid artery is a well characterized model for studying this change in phenotype in vivo. Previous work on cellular proliferation after arterial injury showed that smooth muscle cell proliferation reaches a maximum in the medial layer at 48 h and a maximum in the intimal layer at 96 h and declines thereafter (38). We therefore studied r-SmLIM mRNA levels in rats at 2, 5, and 8 days after balloon injury of the carotid artery (Fig. 7B). SmLIM mRNA levels decreased by more than 60% after day 2 in comparison with control and remained at this level through day 8. These data suggest that r-SmLIM mRNA decreases in response to smooth muscle cell proliferation and dedifferentiation both in vitro and in vivo.

Developmental Regulation of r-SmLIM mRNA Expression—The data described so far suggest that SmLIM is expressed preferentially in vascular tissue and that its levels are affected by the differentiation state of VSMCs. To determine whether SmLIM expression is regulated during development, we isolated total RNA from undifferentiated embryonic stem cells and whole mouse embryos at days 7.5–16.5 post coitum (p.c.). We found that SmLIM expression was indeed regulated developmentally (Fig. 8). Expression was highest during the late primitive streak stage (day 7.5 p.c.), the point at which the embryonic and extraembryonic circulations begin to develop (39, 40). SmLIM expression decreased rapidly at subsequent time points. By normalizing the data to the hybridization signal value at 7.5 days p.c., we found that relative mRNA expression decreased by 40% at 8.5 days p.c. and by approximately 80% at 9.5–16.5 days p.c.

DISCUSSION

We have isolated a developmentally regulated nuclear LIM protein, SmLIM, from a rat smooth muscle cell library. SmLIM is expressed preferentially in arterial smooth muscle cells, and in response to external cues that promote smooth muscle cell proliferation and dedifferentiation, SmLIM mRNA is down-regulated.

SmLIM is a highly conserved, two-LIM-domain nuclear protein of the LIM-only class (Figs. 1–3). Other members of this class include RBTN2, MLP, and CRP. Like SmLIM, RBTN2 and MLP are nuclear proteins with two LIM domains, and they are highly conserved across species (11, 12, 41). CRP proteins also have two LIM domains and show high cross-species conservation (37, 42). Sequence comparisons of SmLIM and CRP suggest that the two gene families are related yet distinct (Fig. 2). In contrast with SmLIM, which is a nuclear protein (Fig. 3), CRP has been localized to the cytoskeletal adhesion plaques (13, 15). Moreover, h-SmLIM localizes to chromosome 3 (Fig. 4), whereas h-CRP localizes to chromosome 1 (43). Finally, Northern analysis of r-CRP tissue distribution showed that the size of its mRNA and pattern of expression were distinct from those of r-SmLIM (data not shown). Taken together, these data indicate that SmLIM and CRP are distinct LIM proteins. While this manuscript was in preparation, Weiskirchen et al. (42) reported the cloning of the chicken CRP2 gene. Sequence comparisons suggest that CRP2 is the avian homologue of SmLIM.

Although SmLIM is highly expressed in smooth muscle cells, it is not expressed in striated muscle cells (Fig. 5). This pattern is in contrast with that of MLP, which is expressed only in the heart and skeletal muscle (11). When a full-length MLP probe...
was hybridized to total RNA from aorta and cultured VSMCs, we were unable to detect a message (data not shown). Thus, the expression of the two LIM proteins is distinct within the myogenic cell lineage. Arber et al. (11) have shown that MLP may play an essential role in striated muscle differentiation. Perhaps SmLIM plays an analogous role in VSMCs.

SmLIM mRNA is expressed preferentially in tissue containing vascular smooth as opposed to nonvascular smooth muscle cells (Fig. 5). As such it joins two other recently identified genes, SM22α and gax (44, 45), expressed highly in VSMC. However, some differences exist in their patterns of expression in tissue. For example, in addition to aorta, SM22α is highly expressed in uterus and intestine (45), whereas SmLIM is not.

**FIG. 6. In situ analysis of r-SmLIM expression in rat vascular tissue.** r-SmLIM mRNA was assayed with 35S-UTP-labeled antisense (left) and sense (right) cRNA probes. Top panels, aorta (Ao), small artery (Ar), and vein (V) at low magnification (× 200). Bottom panels, aorta (Ao) at high magnification (× 600).

**FIG. 7. Down-regulation of SmLIM by growth factor and vascular injury.** A, decrease in r-SmLIM mRNA expression in response to PDGF-BB treatment. Rat aortic smooth muscle cells were made quiescent by incubation in low serum medium (DME plus 0.4% calf serum) for 48 h. Cells were then treated for the indicated times with PDGF-BB (20 ng/ml). Northern analysis was performed with 10 μg of total RNA/lane. After electrophoresis, RNA was transferred to nitrocellulose filters and hybridized with a 32P-labeled r-SmLIM probe. A single r-SmLIM transcript is visible at 1.0 kb. Filters were hybridized with 18S to verify equivalent loading. B, decrease in r-SmLIM mRNA expression after balloon injury in rat carotid arteries. Northern analysis was performed with 20 μg of total RNA/lane at 2, 5, and 8 days after injury. A single r-SmLIM transcript is visible at 1.0 kb. Filters were hybridized with 18S to verify equivalent loading.

**FIG. 8. Developmental regulation of SmLIM mRNA expression.** Total RNA isolated from undifferentiated embryonic stem cells (ES) and mouse embryos days 7.5-16.5 p.c. Northern analysis was performed with 10 μg of total RNA/lane. After electrophoresis, RNA was transferred to nitrocellulose filters and hybridized with a 32P-labeled r-SmLIM probe. A single r-SmLIM transcript is visible at 1.0 kb. Filters were hybridized with 28S to verify equivalent loading.

Gax expression is not detected in intestine but is detected to a high degree in heart (44). By comparison, SmLIM expression appears to be more restricted to aorta. Furthermore, SmLIM is expressed preferentially in arterial as opposed to venous tissue. Arteries and veins have been shown to respond differently to injury (46) and various pharmacological manipulations (47-49); these observations suggest that smooth muscle cells may be fundamentally different in the two tissue types. To our knowledge the pattern of preferential expression in arterial but
not venous smooth muscle cells is unique to SmLIM. SmLIM expression is highest in the adult aorta and correlates with the level of smooth muscle cell differentiation, indicating that its embryonic expression is highest during periods critical for vascular development.

The LIM domain functions as a modular protein-binding interface (52). For example, the LIM-only protein RBTN2 binds to the basic helix-loop-helix protein tal-1 (53), an interaction thought to be critical in regulating red blood cell development. Homozygous deletion of either RBTN2 or tal-1 results in absence of red blood cell formation (12, 54). Similarly, it has been suggested that the effect of the LIM-only protein MLP on myoblast differentiation may be as a cofactor regulating muscle-specific gene expression. Identification of the interaction partner(s) of SmLIM may yield important information about other factors involved in smooth muscle cell development and differentiation.

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Page 10197, Fig. 4: The signal indicated by an arrow in the BIOS somatic cell hybrid blot was generated by a pseudogene on chromosome 3. We have since correctly mapped the human SmLIM gene to chromosome 12 by PCR analysis against a Gene-Bridge 4 radiation hybrid panel. This localization is consistent with the localization reported by Weiskirchen et al. (Weiskirchen, R., Erdel, M., Utermann, G., and Bister, K. (1997) Genomics 44, 83–93).

Page 10197, Fig. 5, legend: The last sentence should read: “A 1.0-kb transcript is shown.”

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A novel growth-promoting factor derived from fetal bovine cartilage, chondromodulin II. Purification and amino acid sequence.

Yuji Hiraki, Hiroyuki Inoue, Jun Kondo, Akihito Kamizono, Yoshino Yoshitake, Chisa Shukunami, and Fujio Suzuki

The sequence for chondromodulin II can be obtained from the PIR data base, accession number JH0270, rather than from GenBank™ as stated in the article.

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Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for e-Jun NH2-terminal kinase in ventricular muscle cells.

Yibin Wang, Bing Su, Valerie P. Sah, Joan Heller Brown, Jiahuai Han, and Kenneth R. Chien

Page 5425, Fig. 4: An incorrect image has been used as panel b. The correct figure is shown below. This does not alter our interpretations or conclusions of the published study.

![MKK6bE](image1.png) ![MKK7D](image2.png) ![MKK3bE](image3.png)  

**MKK6bE + 7D**  **MKK3bE + 7D**

**FIG. 4.** Co-activation of JNK and p38 pathways led to suppression of hypertrophy and induction of cytopathic response and cell death. Cardiac myocytes were either infected with viral vectors expressing MKK6bE (a), MKK7D (b), or MKK3bE (c) alone or co-infected with vectors expressing MKK6bE and MKK7D (d) or MKK3bE and MKK7D (e). The cellular morphology was examined and photographed under light microscopy.

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