Embryonic Expression Suggests an Important Role for CRP2/SmLIM in the Developing Cardiovascular System

Mukesh K. Jain, Saori Kashiki, Chung-Ming Hsieh, Matthew D. Layne, Shaw-Fang Yet, Nicholas E.S. Sibinga, Michael T. Chin, Mark W. Feinberg, Ian Woo, Richard L. Maas, Edgar Haber,† Mu-En Lee

Abstract—Proteins of the LIM family are critical regulators of development and differentiation in various cell types. We have described the cloning of cysteine-rich protein 2/smooth muscle LIM protein (CRP2/SmLIM), a LIM-only protein expressed in differentiated vascular smooth muscle cells. As a first step toward understanding the potential functions of CRP2/SmLIM, we analyzed its expression after gastrulation in developing mice and compared the expression of CRP2/SmLIM with that of the other 2 members of the CRP subclass, CRP1 and CRP3/MLP. In situ hybridization in whole-mount and sectioned embryos showed that CRP2/SmLIM was expressed in the sinus venosus and the 2 cardiac chambers at embryonic day 9. Vascular expression of CRP2/SmLIM was first seen at embryonic day 10. At subsequent time points, CRP2/SmLIM expression decreased in the heart but remained high in the vasculature. CRP1 was expressed both in vascular and nonvascular tissues containing smooth muscle cells, whereas CRP3/MLP was expressed only in tissues containing striated muscle. These patterns of expression were maintained in the adult animal and suggest an important role for this gene family in the development of smooth and striated muscle. (Circ Res. 1998;83:980-985.)

Key Words: muscle, cardiac ■ muscle, smooth ■ development, mouse ■ hybridization, in situ ■ protein, zinc-finger

The LIM motif defines a unique, double–zinc-finger structure found in proteins critical to cellular determination and differentiation.1,2 So far, 4 classes of LIM proteins have been described. Class 1 proteins (LIM-HD) contain 2 LIM domains and a homeodomain. Lin-11, Isl-1, and Mec-3, the first LIM proteins identified, belong to this group.3–5 Class 2 proteins (LIM only) contain 1 or more LIM domains but lack the homeodomain.1,6–9 Class 3 proteins contain C-terminal LIM domains, and most members of this class are cytoplasmic proteins.10 Class 4 proteins do not fit into any of the other LIM classes; some, for example, contain a protein kinase domain (LIM-K).11,12

All 4 classes of LIM proteins are important in the development and function of specific cell types. The LIM-HD protein Lhx3 is critical for pituitary organogenesis, as targeted disruption of the Lhx3 gene eliminates all pituitary cell lineages except the corticotrophs.13 The LIM-only protein RBTN2 is essential for erythroid development, because a homozygous null mutation in RBTN2 leads to failure of yolk sac erythropoiesis and embryonic death.7 The LIM-K protein LIM-kinase1 has been implicated in impaired visuospatial constructive cognition.14

The cysteine-rich protein (CRP) family is a subclass of the LIM-only (class 2) proteins. Members include CRP1, CRP2/smooth muscle LIM protein (SmLIM), and CRP3/muscle LIM protein (MLP). CRP1 has been identified in avian tissues of the gut containing smooth muscle16 and implicated in muscle differentiation.17 CRP3/MLP was first described as a nuclear protein expressed principally in the heart and skeletal muscle of rodents. Overexpression of CRP3/MLP in cultured myoblasts augments differentiation, which suggests that this gene serves as a positive regulator of myogenesis.9 The importance of CRP3/MLP in striated muscle development was demonstrated recently in mice bearing a targeted disruption of the gene.18 CRP3/MLP-deficient animals exhibited profound defects in cardiac as well as skeletal muscle.

We cloned CRP2/SmLIM by homology screening. In adult animals, CRP2/SmLIM was expressed principally in the smooth muscle cells of blood vessels. Within the vasculature, it was expressed preferentially in arterial as opposed to venous tissue. CRP2/SmLIM can localize to both the nuclear and the cytoplasmic compartments.16 CRP2/SmLIM mRNA is downregulated markedly after smooth muscle cell dedifferentiation (in vitro and in vivo)9 and may play an important role.
role in vascular smooth muscle development and differentiation.

To understand the role of CRP2/SmLIM in cardiovascular development, we conducted a detailed spatial and temporal analysis of its expression during mouse embryogenesis and compared the pattern with those of the other 2 members of the CRP family, CRP1 and CRP3/MLP. Our data suggest an important role for this gene family in the development of smooth and striated muscles.

Materials and Methods

In Situ Hybridization on Whole-Mount and Sectioned Embryos

In situ hybridization was performed on whole-mount mouse embryos as described. Sense and antisense digoxigenin-labeled RNA probes were prepared from mouse CRP2/SmLIM cDNA inserts in pCRII. Embryos were fixed in MEMFA buffer (100 mmol/L MOPS [pH 7.4], 2 mmol/L EGTA, 1 mmol/L MgSO₄, and 3.7% formaldehyde) and then stored in 100% methanol at −20°C. After rehydration, embryos were washed at room temperature with 3 changes of detergent (150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L EDTA, and 50 mmol/L Tris-HCl) (30 min/wash) and treated with proteinase K (10 μg/mL) for 5 minutes. Hybridization was carried out overnight at 50°C. After high-stringency washes and treatment with RNase, the embryos were stained with an alkaline phosphatase–coupled antidigoxigenin antibody (Boehringer Mannheim).

To obtain tissue for in situ hybridization at embryonic day (E) 9, we purchased mouse embryo slides from Novagen. For all other time points, embryos were dissected from timed, pregnant CD-1 mice (Charles River Laboratories). Embryos were fixed in 4% paraformaldehyde in PBS. After dehydration and embedding in paraffin, embryos were sectioned at a thickness of 5 μm. Sense and antisense riboprobes labeled with 35S-labeled UTP were transcribed for each gene (CRP1, CRP2/SmLIM, and CRP3/MLP), and hybridization was carried out as described. Tissue sections were autoradiographed on Kodak NTB2 emulsion (Eastman Kodak) for 4 to 8 days at 4°C.

CRP1 and CRP3/MLP cDNAs were amplified from rat aortic smooth muscle cell RNA and mouse fetal heart RNA, respectively. The forward primer 5′-CCAAACTGGGGAGGAGGC-3′ and the reverse primer 5′-CTCTGAATGGACCAAGGC-3′ were used to amplify a 600-bp fragment of CRP1 by reverse transcription and polymerase chain reaction. The forward primer 5′-GAGGCTTCCATGCACCCG-3′ and the reverse primer 5′-CTCTCCACCCCAAAATAG-3′ were used to amplify a 799-bp fragment of CRP3/MLP. The identity of the PCR products was confirmed by nucleotide sequencing. The CRP2/SmLIM cDNA was obtained as described.

Northern Analysis

Various tissues were obtained from CD-1 mice. Total RNA was obtained by guanidinium isothiocyanate extraction and centrifugation through cesium chloride. All RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to a nitrocellulose filter, after which it was hybridized with random-primed, α–32P-labeled probes. Hybridized filters were washed in 30 mmol/L sodium chloride, 3 mmol/L sodium citrate, and 0.1% sodium dodecyl sulfate at 55°C. Autoradiography was performed with Kodak XAR film at −80°C. To correct for differences in loading, blots were hybridized with an oligonucleotide probe complementary to 28S rRNA. Filters were scanned and radioactivity was measured on a PhosphorImager running the ImageQuant software (Molecular Dynamics).

Results

Cardiac Expression of CRP2/SmLIM During Mouse Embryogenesis

As a first step toward understanding the potential functions of CRP2/SmLIM in cardiovascular development, we performed a detailed analysis of its expression during mouse gestation. At E9, the developing linear heart assumes an S shape and shows evidence of regionalization along its length. The heart is divided into a common atrial chamber that receives blood from the right and left sinus venosus and a common ventricular chamber that is contiguous to the outflow tract. Whole-mount and section in situ hybridization at E9 revealed that CRP2/SmLIM expression occurred throughout the developing heart (Figure 1A). Expression was high in the sinus venosus (Figure 1A and 1B) and was visible in the developing ventricle (Figure 1C). Expression was highest in the outflow tract (Figure 1C).

At E10, CRP2/SmLIM expression was maintained in the atrium but decreased slightly in the ventricle (Figure 2A and 2B) in comparison with expression at E9. By E12 (Figure 2C), this difference in CRP2/SmLIM expression in the cardiac chambers became more apparent. Although at E12 CRP2/SmLIM expression was maintained in the atrium (Figure 2C), it decreased markedly in the ventricle. This pattern of differential CRP2/SmLIM expression was also maintained in the heart at E15 (Figure 3A and 3B) and in adults (Figure 6).

Vascular Expression of CRP2 During Mouse Embryogenesis

At approximately E8, endothelial cells amalgamate to form features of the embryonic vasculature such as the paired dorsal aortae. Although the precise timing of smooth muscle cell recruitment to the developing vasculature is unclear, it probably occurs by E10.5, because at this point expression of highly specific markers of differentiated smooth muscle cells is visible. CRP2/SmLIM expression was first visible within...
the vasculature at E10 (Figure 2A and 2B, dorsal aorta, and umbilical vessel). By E15, expression occurred in several additional vessel beds, such as the mesenteric vasculature (Figure 3C) and the pulmonary and cerebral vasculature (not shown).

Expression of CRP1 and CRP3/MLP During Mouse Embryogenesis

We performed a detailed analysis of CRP1 and CRP3/MLP expression during mouse development to compare and contrast their expression with that of CRP2/SmLIM. CRP1 expression was robust in both the atrial and the ventricular chambers at E9 (Figure 4A). Furthermore, CRP1 expression was visible at this point in the paired dorsal aortae (Figure 4A and 4B) and myotome (Figure 4A). At E10, CRP1 expression was maintained in the heart and vasculature (Figure 4C), and at E15, a point at which the primitive gut undergoes spatial development and differentiation, CRP1 expression was robust in the esophagus and the gut (Figure 4D). CRP1 expression was also visible in both the parenchyma and blood vessels of the lung (Figure 4D and not shown).

Intense CRP3/MLP expression occurred at E9 in the developing atrial and ventricular chambers of the heart (Figure 5A and 5C). Cardiac expression of CRP3/MLP remained high throughout gestation (Figure 5B and 5C). Weak expression of CRP3/MLP was also visible in the myotome at E10 (data not shown). By E15, CRP3/MLP was visible in the skeletal musculature of the back, chest wall, and diaphragm (Figure 5C).

CRP Gene Expression in Adult Mice

To compare and contrast expression of the 3 CRP genes in adult mice, we performed Northern analyses with a variety of tissues (Figure 6). The expression pattern of each gene at E15 was similar in adults. CRP1 was expressed in both arterial (aorta) and venous (vena cava) tissues, whereas CRP2/SmLIM was expressed principally in arterial tissue. Both genes were expressed at low levels in the atrial chambers of the heart and at minimal levels in the ventricular chambers (Figure 6). In contrast, CRP3/MLP was expressed at high levels in the 2 heart chambers. CRP1 was expressed highly in all tissues containing nonvascular smooth muscle, such as uterus and colon (Figure 6). CRP2/SmLIM was expressed in uterus and colon but at much lower levels. In lung, CRP1 mRNA expression was high, whereas that of CRP2/SmLIM was minimal (Figure 6).

Discussion

We show in this report that CRP2/SmLIM is expressed in the cardiovascular system at early time points during mouse development. As gestation progresses, CRP2/SmLIM expression decreases in the heart (particularly in the ventricle) but remains high in the vasculature. A comparison of CRP2/
SmLIM expression with that of the other 2 members of the CRP family reveals distinct patterns of tissue distribution during cardiovascular development. Although all 3 CRP genes are expressed early in the developing heart, only CRP3/MLP is expressed at high levels through the end of gestation and into adulthood (Figures 5 and 6). Expression of both CRP1 (Figure 4) and CRP2/SmLIM (Figures 2 and 3) is high in a number of vascular beds. In contrast with CRP2/SmLIM expression, however, CRP1 expression is less restricted. Robust expression of CRP1 also occurs in nonvascular tissues containing smooth muscle, such as the gut (Figure 4) and the uterus (Figure 6).

Our results agree in general with the recent observations of Louis et al. on the pattern of CRP gene expression during chick development. Using CRP isoform–specific antibodies, Louis et al. analyzed a number of tissues from 19-day chicken embryos by Western blotting. They found that CRP1 was present predominantly in organs enriched in smooth muscle (arteries, stomach, gizzard, intestine, and colon) and in lung and fibroblasts. CRP2 expression was limited to arteries and fibroblasts, whereas CRP3/MLP was expressed exclusively in heart, crop, and skeletal muscle. An important difference between our observations and those of Louis et al. is that they found no expression of CRP1 or CRP2 in...
embryonic chick hearts. This discrepancy is probably due to the fact that Louis et al.²³ examined heart samples at E19. We examined heart samples at earlier points during mouse embryogenesis (Figures 1 and 4).

Cardiac expression of CRP2/SmLIM begins early in gestation. CRP2/SmLIM shares this feature with other smooth muscle markers, such as α-actin, calponin, and SM22α, the transient expression of which occurs early during cardiac morphogenesis.²²,²⁴,²⁵ Given that the heart begins as a tubular structure with rhythmic contractions, it has been hypothesized that embryonic cardiomyocytes traverse a smooth muscle cell-like phenotype during the development of the heart.²⁴,²⁷ Our observations support this hypothesis. The functional significance of an overlap in the genetic programs of cardiac cells and smooth muscle cells early in development is still not clear. However, even transient gene expression in embryonic cardiomyocytes could be very important to the normal development of the heart.

We have reported elsewhere that CRP2/SmLIM is expressed principally in the vasculature of the adult rat. The data presented here extend our earlier observations by showing that CRP2/SmLIM is expressed in the mouse vasculature as early as E10 (Figure 2). Thus, CRP2/SmLIM is among the earliest smooth muscle genes expressed in the developing aorta. One sensitive indicator of differentiated smooth muscle cells is smooth muscle myosin heavy chain,²² which is expressed in the mouse aorta at E10.⁵² CRP2/SmLIM is expressed in the vasculature (at E10) shortly before smooth muscle cells are thought to assume a differentiated phenotype.

The function of genes of the CRP family in the development of the heart and vasculature is still being elucidated. Our observations support this hypothesis. The functional significance of an overlap in the genetic programs of cardiac cells and smooth muscle cells early in development is still not clear. However, even transient gene expression in embryonic cardiomyocytes could be very important to the normal development of the heart.

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