Up-Regulation of *cyr61* in Vascular Smooth Muscle Cells of Spontaneously Hypertensive Rats

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SUMMARY: In the present study, we applied a fluorescent differential display method to mRNAs from aortae of spontaneously hypertensive rats (SHRs), stroke-prone spontaneously hypertensive rats (SPSHRs), and their parental strain, Wistar Kyoto rats (WKYRs), to identify the genes involved in the development of hypertension. Through this screen we came across a gene that is consistently up-regulated in hypertensive rats. Nucleotide sequence determination of the corresponding cDNA revealed that the gene is the rat orthologue of *cyr61*. Northern blot analysis showed that *cyr61* expression increases in SHR and SPSHR before the onset of hypertension and is sustained thereafter at higher levels than in age-matched WKYRs. In situ hybridization analysis demonstrated that *cyr61* is expressed strongly in smooth muscle cells (SMCs) in media of SHR and SPSHR but not WKYR aorta. Fluorescent in situ hybridization mapped the *cyr61* gene to rat chromosome 1p12–13, which is located in close proximity to a recently defined quantitative trait locus including NHE3 Na^+/H^+ exchanger. Overexpression of the *cyr61* gene in stably transfected rat SMC line A7r5 caused rather inhibitory effects on the proliferation and DNA and protein synthesis. Our results thus demonstrate for the first time that *cyr61* can also act as a growth inhibitor in SMC of genetically hypertensive rats. This may reveal a new route for investigation of the pathogenesis of hypertension. (*Lab Invest 2003, 83:973–982*).

*E*ssential hypertension is the most prevalent cardiovascular disorder and has been recognized as a polygenic disease that probably occurs due to complex gene-to-gene and environment-to-gene interactions and their disturbances (Hamet et al, 1998). Several causal genes have been suggested as candidates for essential hypertension. However, the relationship between these genes and systemic hypertension is not yet fully understood; association and linkage studies have not necessarily given consistent results (Brand et al, 1998; Kunz et al, 1997).

The spontaneously hypertensive rat (SHR) was established as an animal model for essential hypertension by recurrent selective breeding of Wistar rats (Okamoto and Aoki, 1963). SHR and its normotensive

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counterpart would provide an opportunity to compare gene expressions or genetic changes between normal and hypertensive states to gain insights into the pathogenesis of essential hypertension. In this study, we applied the mRNA differential display method (Ito and Sakaki, 1999) to identify genes that are differently expressed in SHR and came across the rat homolog of the *cyr61* gene, whose expression was consistently increased in hypertensive rats. The *cyr61* gene encodes a secreted cysteine-rich heparin-binding protein and belongs to a growing family of cysteine-rich, secreted growth regulators called Cyr61/Cef10 Nov (CCN) family (Bork, 1993) including connective tissue growth factor (CTGF) (Bradham et al, 1991), Cyr61/Cef10 (O'Brien et al, 1990), and the *Nov* gene (Martinerie et al, 1996). The *cyr61* gene was found to be overexpressed in smooth muscle cells (SMCs) of the hypertensive rat aortae and can also act as a growth inhibitor in SMC.

Results

Fluorescent Differential Display (FDD) Screening of Genes Differentially Expressed in Hypertensive Versus Normotensive Rat Aortae

As shown in Figure 1A, it was confirmed that as SHRs aged, systolic blood pressure increased, reaching a plateau of about 230 mmHg. Stroke-prone spontaneously hypertensive rats (SPSHRs) also exhibited in-
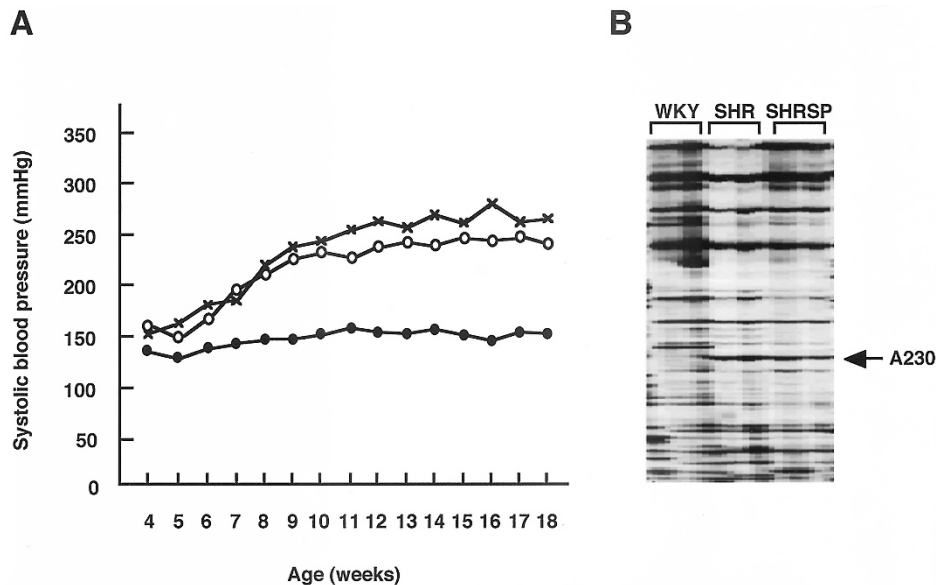
A

B

Figure 1.

Identification of mRNA activated in the aorta of hypertensive rats. (A) The development of hypertension in spontaneously hypertensive rats (SHRs) and stroke-prone spontaneously hypertensive rats (SHRSP). Line graphs show the mean values of systolic blood pressure in SHR (○), SHRSP (X) and Wistar Kyoto rat (WKYR) (●) ($n = 20$). (B) Differential display analysis of RNAs from aortae. 2.5-μg of total RNAs isolated from 6-week-old rats were used for the fluorescent differential display analysis. RT-PCR products were run in duplicate on adjacent lanes. *Arrow* indicates the product that represents the mRNA species designated A230. Essentially the same results were obtained in several experiments using different RNA samples obtained by independent isolations.

creased systolic blood pressure but from a younger age in comparison with SHR, reaching a higher level of around 250 mmHg. At 12 weeks after birth, the values recorded in SHRs and SPSHRs were 1.6- and 1.8-times higher than in Wistar Kyoto rats (WKYRs), respectively. At 6 weeks, however, there were no significant differences in systolic blood pressure among the three strains (Fig. 1A). RNAs were thus isolated from aortae of 6-week-old SHRs, SPSHRs, and WKYRs and underwent FDD analysis to identify the genes that were differentially expressed in the hypertensive and normotensive rats. Fifteen candidate cDNAs were identified, and two of them had the same sequence of 296 bp in length, which was designated as A230. Figure 1B shows a typical FDD pattern, where the band corresponding to A230 was clearly marked in SHRs and SPSHRs with stronger signal intensities than in WKYR. RNA blot analysis detected a single RNA species that hybridized to the A230 FDD fragment and confirmed its elevation in SHR and SPSHR aortae (data not shown).

Isolation and Sequence Determination of Full-Length A230 cDNA

We performed 5'-rapid amplification of cDNA ends (5'-RACE) using SHR aorta-derived poly(A)$^+$ RNA and A230-specific primers to isolate the full-length cDNA and determined the nucleotide sequence of the resultant 5'-RACE product. The cDNA was 1871-bp long and had a large open reading frame encoding a 379-amino acid protein with a 24–amino acid putative signal peptide (Fig. 2A). Searching of the DDBJ/Gen-Bank/EMBL DNA databases revealed the highest nucleotide sequence homology (94.6%) in the coding

region between the full-length A230 cDNA and mouse *cyr61* cDNA (O'Brien et al, 1990). The deduced A230 protein also exhibited high homologies with mouse and human Cyr61 proteins; identity in amino acids was 93.5% and 87.9%, respectively. Significant homology was also noted with the other members of the CCN family; human CTGF and NovH proteins showed 42% and 38.7% amino acid identity to the deduced A230 protein (Fig. 2A). CCN family proteins are cysteine-rich and have four conserved domains: the IGF binding protein-like domain, the von Willebrand factor type C repeat domain, the thrombospondin type 1 repeat domain, and the COOH-terminal domain (Fig. 2B) (Bork, 1993). The rat A230 protein contained 38 cysteine residues, all of which were completely conserved among these family members and had 40% to 70% sequence identity with human CTGF and NovH in each of the four domains (Fig. 2B). From these results, we concluded that the A230 cDNA is the rat orthologue of *cyr61*, belonging to the *CCN* gene family.

Time Course of cyr61 mRNA Expression

The time course of *cyr61* expression in the hypertensive rat was examined. Total RNAs were isolated from the aortae of 4-, 6-, and 12-week-old hypertensive rats and age-matched WKYRs, and analyzed by Northern blotting using a 1633-bp fragment of *cyr61* cDNA (nucleotides 1–1633) as a probe. As shown in Figure 3, a hybridizing band was marked at 2.6 kb, and the mRNA level increased in SHR and SPSHR compared with that in WKYR. The levels of *cyr61* mRNA were 3.6- (4-week-old SHR) and 4.7- (4-week-old SPSHR), 2.0- (6-week-old SHR) and 1.8- (6-week-old SPSHR), and 1.4- (12-week-old SHR) and 1.9- (12-week-old

Figure 2.
Alignment of the deduced amino acid sequences of rat Cyr61, mouse Cyr61, human Cyr61, human connective tissue growth factor, and NovH proteins (A) and a schematic representation of the domain structure of rat Cyr61 protein (B). (A) Positions of the identical amino acids are shaded. Putative signal sequences are boxed by a *broken line*. The 38 conserved cysteine residues are shown by *asterisks*. (B) Numbers indicate the amino acid identity in percentage between the corresponding domains of rat Cyr61 and the other CCN family members. *IGFBP* = IGF binding protein-like domain; *vWF* = von Willebrand factor; *TSP1* = thrombospondin type 1 repeat domain; *CT* = COOH-terminal domain. The nucleotide sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB015877.

SPSHR) fold higher than those in the age-matched WKYR, respectively (Fig. 3). No statistically significant difference was observed in the *cyr61* mRNA level between SHR and SPSHR.

Tissue Distribution of cyr61 mRNA

The tissue distribution of *cyr61* gene expression was then examined by Northern blotting in 6-week-old

A

	4-week-old	6-week-old	12-week-old
	WKY SHR SHRSP	WKY SHR SHRSP	WKY SHR SHRSP

cyr61

28S rRNA

B

Relative *cyr61* mRNA levels

Figure 3.
Time course changes of *cyr61* expression in the aorta. (A) Northern blot analysis of total RNAs derived from the aorta of 4-, 6-, and 12-week-old rats. Acrydine orange stain of 28S rRNA is shown in the lower panel. (B) Quantification of the *cyr61* mRNA levels. Intensities of the *cyr61* signals were normalized with those of 28S rRNA and are related to the values of Wistar Kyoto rat. Values are expressed as the mean $\pm$ SD ($n = 5$). *$p < 0.05$.

WKYRs. It revealed a relatively wide distribution of *cyr61* expression, but the highest expression was noted in the aorta (Fig. 4). The mRNA of *cyr61* was present at moderate levels in the heart, skeletal muscle, kidney and lung, and at lower levels in the small intestine, adrenal grand, brain, and liver (Fig. 4). This pattern of *cyr61* expression was consistent with the previous reports on mouse and human *cyr61* expressions (Jay et al, 1997; O'Brien et al, 1990; Perbal et al, 1999).
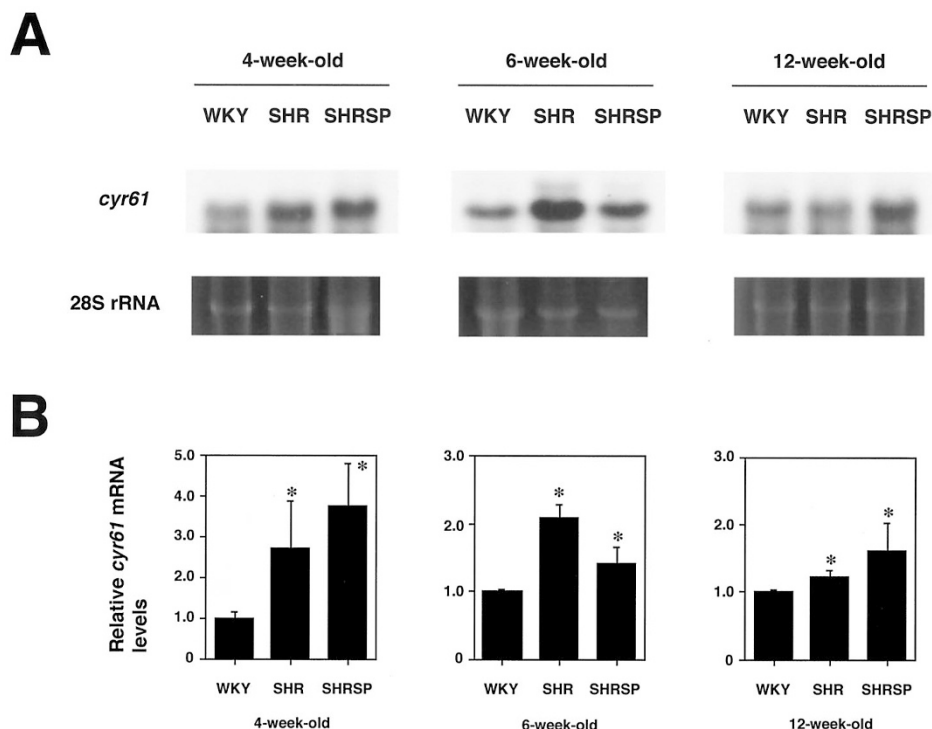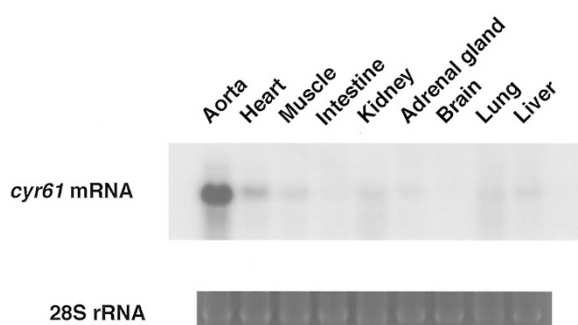

Figure 4.
Tissue distribution of *cyr61* mRNA. Twenty-five μg of total RNAs from the indicated Wistar Kyoto rat tissues were subjected to electrophoresis on a 1.5% agarose gel containing 1.1 M formaldehyde and hybridized with ³²P-labeled rat *cyr61* cDNA probe. 28S rRNA was used as the reference standard.

In Situ Detection and Localization of cyr61 mRNA in Rat Aortae

To identify the cell types that express the *cyr61* gene, we performed an in situ hybridization analysis of the aortae from 6-week-old SHRs, SPSHRs, and WKYRs. In WKYR blood vessels, only weak signals were detected in fibroblasts in the adventitia (Fig. 5A). In contrast, strong *cyr61* mRNA-derived signals were marked in SMC in the medial layer of SHR and SPSHR blood vessels (Fig. 5, C to E). Fibroblasts in SHR and SPSHR aortae showed a moderate expression of *cyr61* mRNA as in the WKYR blood vessels. Microscopic observation disclosed no hyperplasia of SMC in hypertensive rat arteries at this stage (Fig. 5F).

Chromosomal Mapping of Rat cyr61 Locus

Congenic approaches have delimited candidate loci for hypertensive traits in SHR (Hamet et al, 1998; Wakeland et al, 1997). Accordingly, to determine whether the *cyr61* gene resides within these loci, we then mapped the chromosomal localization of rat *cyr61* gene by fluorescent in situ hybridization (FISH) using the *cyr61* genomic fragment as a probe (Fig. 6). Of a total of 100 hybridizing signals observed, 90 (90%, $p < 0.01$) were localized over 1p12–13 (Fig. 6A). Of the 90 chromosome 1 analyzed, 80 had labeling on both chromatids, and 10 were labeled on a single chromatid (Fig. 6B).
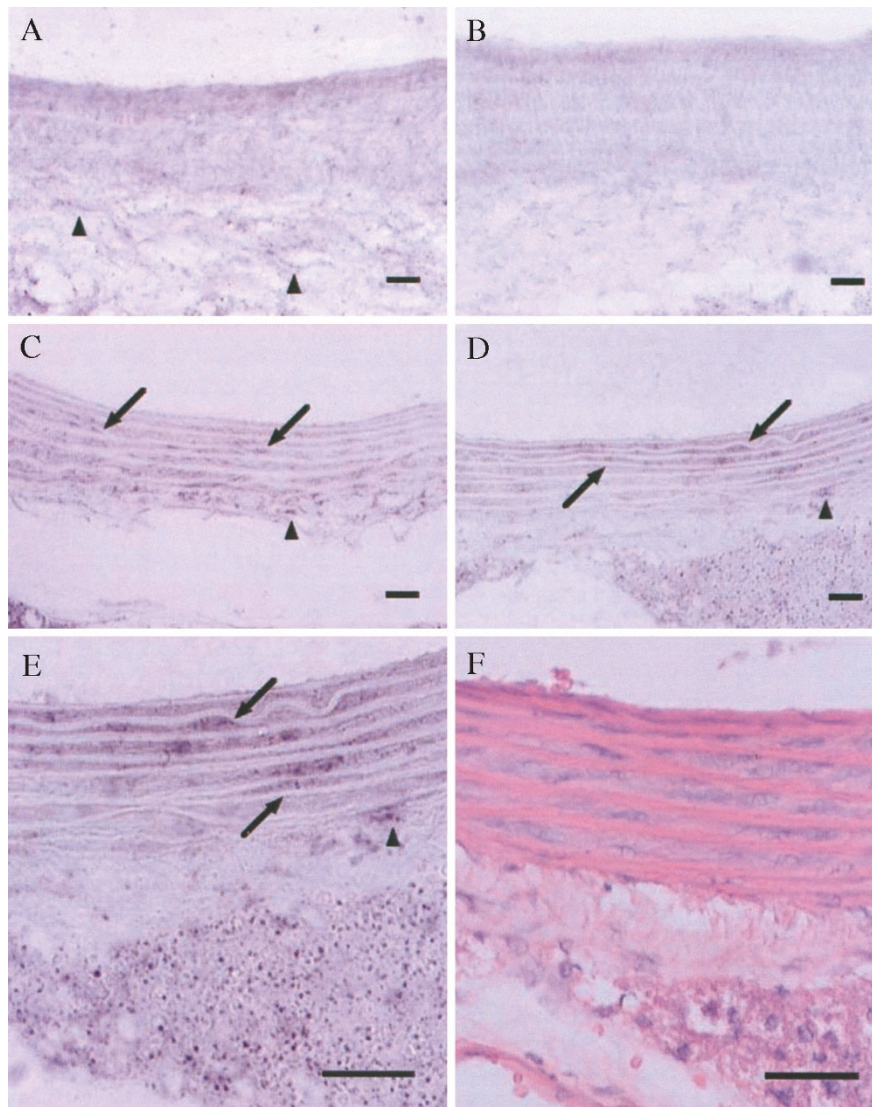
Figure 5.
Localization of *cyr61* mRNA in rat blood vessels. In situ hybridization analysis of the arteries of 6-week-old Wistar Kyoto rats (A), spontaneously hypertensive rats (C), and stroke-prone spontaneously hypertensive rats (D) using digoxigenin (DIG)-labeled *cyr61* antisense riboprobe. (F) Hematoxylin-eosin staining of the SPSHR artery, showing the absence of hyperplasia of smooth muscle cell (SMC). (E) Higher magnification of D. (B) Control experiment with DIG-labeled *cyr61* sense probe; the section was from 6-week-old SPSHR as were D and E. *Arrowheads* indicate *cyr61* mRNA-derived signals in fibroblasts. *Arrows* indicate *cyr61* mRNA-derived signals in SMC. Scale bar indicates 100 μm.

Cyr61 Transfection into SMC

To examine the roles of *cyr61* in the hypertensive process, we established rat SMC line A7r5, which overexpresses Cyr61 proteins. The proteins were recovered in conditioned media of Cyr61-A7r5 cells (see "Materials and Methods" section), and quantitatively bound to and salt-eluted from heparin-Sepharose column (data not shown), being consistent with the previous report (Kireeva et al, 1996). The recombinant Cyr61 was confirmed by Western blot analysis to contain histidine tag at its C-terminal and to migrate to the position of about 36 kd as expected (Fig. 7A). The recombinant Cyr61 proteins were not detected in media from A7r5 cells transfected with the pcDNA3 vector alone.

Effects of Cyr61 Overexpression on A7r5 Cell Proliferation

Cyr61 overexpression showed rather inhibitory effects on cell proliferation and macromolecular synthesis. The growth curve shifted downward in Cyr61-A7r5 cells compared with the A7r5 parental line (Fig. 7B). Thymidine and leucine incorporation was also significantly reduced by Cyr61 overexpression (Fig. 7C). A series of experiments with another *cyr61* overexpressor clone revealed a similar tendency (data not shown).

Discussion

In the present study, an FDD screen led to the isolation of a new rat gene that was differently expressed in the

Figure 6.

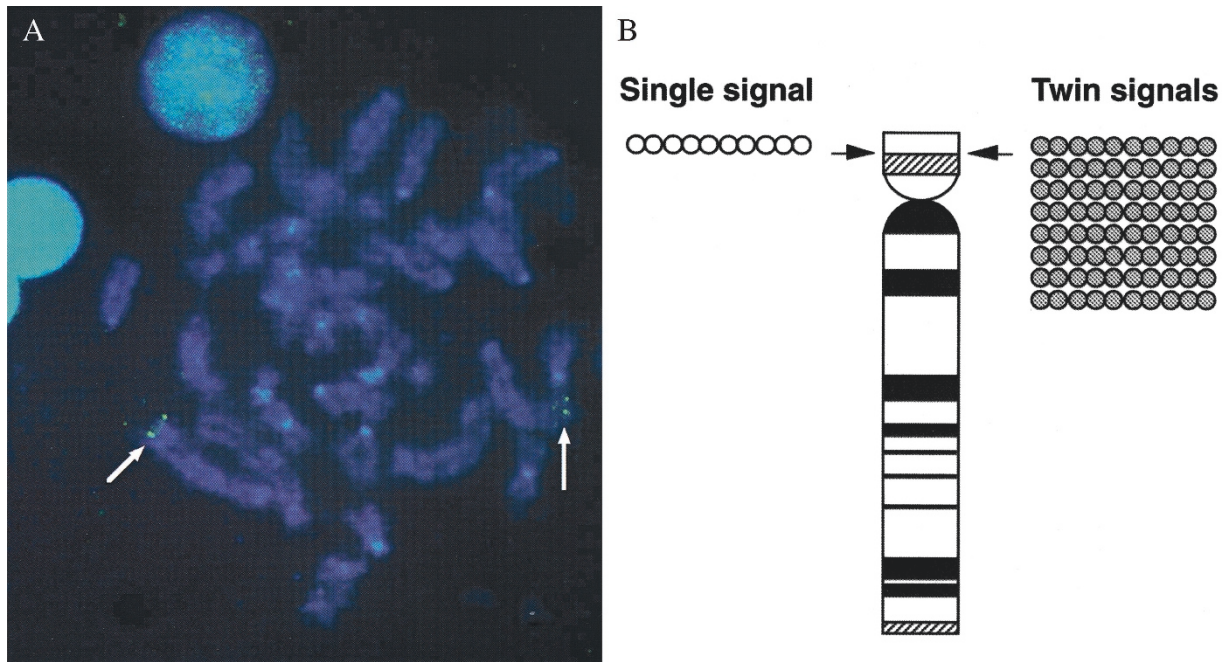Chromosomal localization of rat *cyr61* by fluorescence in situ hybridization. (A) R-banded metaphase chromosomes showing the localization of spots on both chromatids of chromosome 1. Metaphase chromosomes were spread from rat bone marrow cells and hybridized to the biotin-labeled *cyr61*-genomic fragment (4.8 kbp), which was then visualized with fluorescein isothiocyanate–conjugated streptavidin. *Arrows* indicate fluorescent signals on 1p12–13. Q-band–like image of the same metaphase is overlapped. (B) Ideogram depicting the distribution of fluorescent spots over chromosome 1. *Dotted circle* indicates 1 chromosome with twin signals; *open circle* indicates 1 chromosome with a single signal.
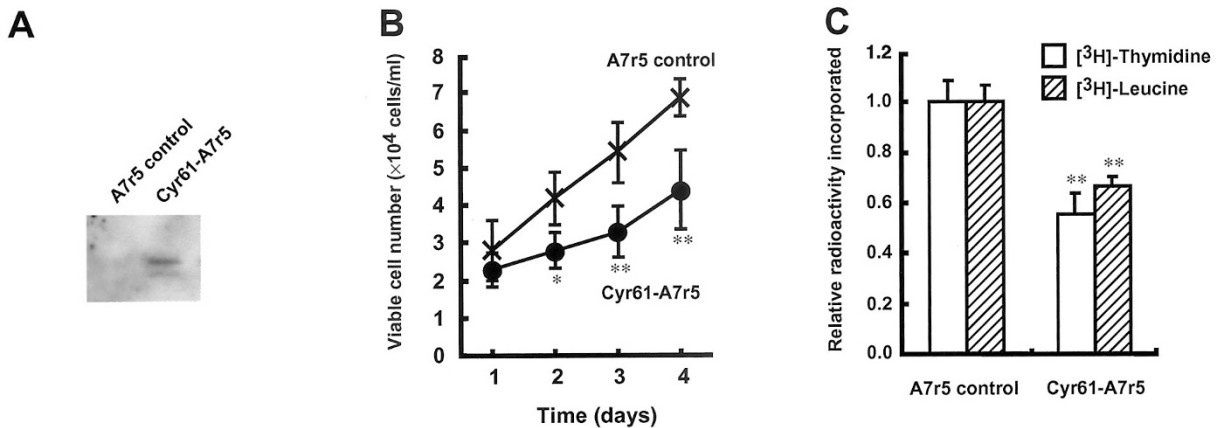
Figure 7.

Overexpression of Cyr61 protein in A7r5 cells. (A) Representative Western blot of Cyr61 protein in A7r5 cells. Conditioned media of A7r5 control cells (Lane 1) or Cyr61-A7r5 cells (Lane 2) were loaded onto a HiTrap Heparin column. Proteins were eluted from the column with 0.4 to 0.6 M NaCl and were analyzed by 10% SDS-PAGE followed by Western blotting with antipenta His antibodies as probe. (B) Effects of Cyr61 overexpression on viable cell number. Results are given as the growth of Cyr61-A7r5 cells (●) and A7r5 control cells (×). Values are expressed as the mean ± SD (*n* = 4). (C) Effects of Cyr61 overexpression on DNA and protein syntheses. Values are expressed as the mean ± SD (*n* = 3). Experimental conditions are described in "Materials and Methods" section. *$p < 0.05$; **$p < 0.01$.

aortae of SHRs and SPSHRs. The isolation and sequence determination of the near full-length cDNA revealed that it was the rat orthologue of *cyr61*. The *cyr61* gene was first identified as a growth factor–inducible immediate early gene in mouse fibroblasts, and so designated because it encodes a secreted cysteine-rich heparin-binding protein (O'Brien et al, 1990). We examined the expression of the *cyr61* gene in the aorta during the course of the development of hypertension. Even before the onset of hypertension,

cyr61 mRNA levels were consistently higher in SHR and SPSHR aortae than in age-matched WKYR aortae (Fig. 3). The blood vessel was found to express *cyr61* at the highest level among the tissues examined (Fig. 4). In situ hybridization study revealed that the *cyr61* mRNA was strongly expressed in SMC at the medial layer of the aorta in SHRs and SPSHRs (Fig. 5). FISH analysis defined the *cyr61* gene locus at rat chromosome 1p12–13, which resides adjacent to a recently defined blood pressure quantitative trait locus having

the candidate gene *Slc9a3* for sodium-hydrogen exchange (Fig. 6) (Saad et al, 2001). *Slc9a3* encodes Na^+/H^+ exchanger 3 (NHE3), which is one of five plasma membrane Na^+/H^+ exchangers and is expressed in the kidney (Tse et al, 1992). In *Slc9a3⁻/⁻* mice, there is a severe absorptive defect in the kidney and intestine, which causes reduced blood pressure (Schultheis et al, 1998). In the present study, we established rat vascular SMC lines that overexpress Cyr61 proteins and showed that Cyr61 rather inhibited SMC proliferation and DNA and protein syntheses (Fig. 7). In contrast to our results, previous studies reported growth factor–induced mitogenic effects of Cyr61 in fibroblasts (Kireeva et al, 1996) and endothelial cells (Kolesnikova and Lau, 1998). Recent studies have shown that *cyr61* expression is induced in arterial SMC following balloon angioplasty (Grzeszkiewicz et al, 2002) and that *cyr61* may regulate the process of wound repair, including angiogenesis, inflammation, extracellular matrix remodeling, and cell-matrix interactions (Chen et al, 2001). Because the expression of *cyr61* has been reported to be under the control of growth factors, among which basic fibroblast growth factor (bFGF) is known as the principal inducer (Lau and Lam, 1999), the possibility that inducible expression of *cyr61* is regulated by growth factors such as bFGF cannot be ruled out. We assayed the bFGF contents in SHR, SPSHR, and WKYR blood vessels and observed no significant change in bFGF levels among them, suggesting that the induction of *cyr61* expression in SMC cannot be primarily ascribed to bFGF. Others also demonstrated that *cyr61* expression in SMC is influenced by blood pressure in vitro (Tamura et al, 2001).

Angiotensin II acts as an important mediator in cardiovascular diseases such as hypertension, congestive heart failure, and renal failure. Recent studies have shown that angiotensin II stimulated *cyr61* expression in SMC via AT_1 receptor signaling (Hilfiker et al, 2002). The fact that in human atherosclerotic lesions Cyr61 expression is colocalized with angiotensin II suggests a new role of *cyr61* in atherosclerosis (Hilfiker et al, 2002). *CTGF*, another member of the CCN family, has been shown to be overexpressed in vascular SMC and in luminal and vasa vasorum endothelial cells of advanced atherosclerotic lesions (Oemar et al, 1997). *CTGF* promotes the growth of fibroblasts (Frazier et al, 1996) and endothelial cells (Shimo et al, 1998) but inhibits the proliferation of SMC (Hishikawa et al, 1999, 2000). The precise mechanism of action of the CCN family members remains to be determined. Further studies on the biological function of *CCN* family members should provide a new clue for understanding the pathogenesis of blood pressure elevation and vascular derangement in hypertension.

In conclusion, the present study has demonstrated for the first time that *cyr61* is overexpressed in hypertensive rat aortae and inhibits cell proliferation and growth of SMC. Despite recent therapeutic advances, essential hypertension continues to be the major problem affecting the progression of atherosclerosis and diabetic angiopathy. The overexpression of *cyr61*

may exert an important role in artery remodeling during the development and establishment of hypertension and thus present a potential target of therapeutic intervention that warrants further investigation.

Materials and Methods

Animals

Male SHRs (SHR/Izm), SPSHRs (SPSHR/Izm), and WKYRs (WKYR/Izm) were obtained from the Disease Model Company-Operative Research Association (Kyoto, Japan). Rats were housed two per container and fed standard rat chow and water. All experiments were conducted in accordance with the institutional guidelines of the Takara-machi campus of Kanazawa University.

Measurements of Blood Pressure

Twenty male rats were used for indirect blood pressure measurements with a UR-5000 instrument (Elquest Corporation, Tokyo, Japan). The tail cuff method was carried out as previously reported (Ikeda et al, 1991). Three consecutive consistent readings were taken in each individual, and the values were averaged.

Isolation of Aortae

Aortae encompassing the beginning of the thoracic segment to the aortic bifurcation were extirpated under anesthesia from 4-, 6-, or 12-week-old SHRs, SPSHRs, and WKYRs and washed with Hank's balanced salt solution. After dissecting off loose periadventitial connective tissues, the aortae were frozen in liquid nitrogen and stored at $-80°$ C until RNA isolation.

Isolation of RNAs

The frozen aortae were disrupted in TRIZOL Reagent (Invitrogen Corporation, Carlsbad, California) using a Polytron homogenizer (KINEMATICA AG, Littau/Luzern, Switzerland) at the maximum speed for 30 seconds, and total RNAs were isolated according to the manufacturer's instructions. For 5'-RACE, poly(A)⁺ RNAs were isolated from the total RNAs using Quick-Prep mRNA Purification Kit (Amersham Biosciences Corporation, Piscataway, New Jersey). The RNA concentration was determined by measuring absorbance at 260 nm.

FDD Analysis

FDD analysis was performed according to the method developed by others (Ito and Sakaki, 1999). Total RNAs (2.5 μg) from aortae of 6-week-old SHRs, SPSHRs, and WKYRs were reverse-transcribed with Texas red-labeled 3'-anchored oligo-dT primer (Texas red–T_{15}N; N is either A, C, or G) and SuperScript II RNase H^- Reverse Transcriptase (Invitrogen Corporation), and the resultant reverse-transcribed products were used as templates for PCR. PCR was performed

with an arbitrary primer (GenHunter Corporation, Nashville, Tennessee), the same 3'-anchor primer and GeneTaq Polymerase (Nippon Gene, Toyama, Japan). The amplification products were separated by denaturing 6% polyacrylamide gel electrophoresis, and the patterns of DNA fragments were analyzed with a Hitachi SQ-5500 sequencer (Hitachi Inc., Tokyo, Japan). The reproducibility of the experiment was confirmed in several independent experiments.

Cloning and Sequencing of cDNA Fragments of Interest

cDNAs that gave different signal intensities on FDD were recovered from the gel by excision and extraction with hot water. The recovered DNA was then re-amplified using the same set of primers and ligated to a pCR2.1 vector (Invitrogen Corporation) to transform *Escherichia coli* XL1-blue cells (Stratagene, La Jolla, California). Recombinant plasmid DNA was purified from the bacterial culture using Flexiprep Kit (Amersham Biosciences Corporation). Nucleotide sequences of cloned cDNA fragments were determined with the Hitachi SQ-5500 sequencer or with an Applied Biosystems 377 DNA sequencer (The Perkin-Elmer Corporation, Foster City, California) by the dideoxy chain termination method (Sanger et al, 1977).

Northern Blot Analysis

Twenty-five μg of total RNAs from aortae of 4-, 6-, and 12-week-old WKYRs, SHRs, and SPSHRs underwent agarose gel electrophoresis and filter blot hybridization as described previously (Yonekura et al, 1999). The cDNA fragments were labeled by the random priming method using [α-^{32}P]dCTP (NEN Life Science Products, Inc., Boston, Massachusetts). The radioactivities of the hybridized bands were measured with a Fujix BAS 1000 BioImage analyzer (Fuji PhotoFilm Company Ltd., Hamamatsu, Japan). The equivalency and intactness of the loaded RNA were assessed by acrydine orange staining of 28S and 18S ribosomal RNAs.

Isolation of Full-Length cDNA

To obtain the full-length cDNA, the 5'-RACE was carried out using Marathon cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, California) and Klentaq polymerase mix (BD Biosciences Clontech) with a primer 5'-TCC ACT GGG GAA GGC AGG AGA G-3', the complement of the 5'-terminal region of the FDD-selected cDNA fragment. The amplified DNA fragments were cloned into the plasmid vector pCR2.1 and purified for sequence analysis. Both strands of the cDNA were sequenced. The cDNA sequence was analyzed for similarity with nucleotide sequences deposited in DDBJ/EMBL/GenBank databases by the BLAST algorithm.

mRNA Localization by In Situ Hybridization

Aortae from 6-week-old SHRs, SPSHRs, and WKYRs were analyzed by an in situ hybridization technique using digoxigenin (DIG)-labeled RNA probes with slight modification (Oemar et al, 1997). In brief, tissues were frozen in liquid nitrogen, and serial 10-μm cryosections of thoracic arteries were mounted on silane-coated slides and fixed in 4% buffered paraformaldehyde for 10 minutes. The sections were carbethoxylated with 0.1% diethylpyrocarbonate in PBS twice for 15 minutes and subjected to hybridization. Riboprobes were prepared from a plasmid pBS (Stratagene) carrying a 586-bp *Eco*RI/*Pst*I fragment of the cloned rat *cyr61* cDNA (439–1024 nucleotide). The plasmid was linearized by digestion with *Eco*RI (to prepare the antisense riboprobe) or *Hin*dIII (to prepare the control sense probe). The probes were labeled with DIG-11-UTP using a DIG RNA-labeling kit (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Sections were hybridized with DIG-RNA probes, and the presence of riboprobe-mRNA duplex was visualized with NBT/BCIP. The sections were mounted with Crystal/Mount (Biomeda Corporation, Foster City, California) and observed under a light microscope.

FISH Analysis of Rat Chromosomes

The FISH procedure was carried out essentially as described previously (Nakagawara et al, 1995). The DNA probe used for FISH was prepared as follows. Because the nucleotide sequence of the rat *cyr61* gene was not known and because rat and mouse genes generally show strong structural conservation, we designed several sets of PCR primers based on the mouse *cyr61* genomic sequence (Latinkic et al, 1991) and used them to amplify the rat equivalent with high molecular weight DNA from the liver of WKYR as a template. A set of primers, 5'- GTT ACG TCT GGT GTC TGA TTC ACC ATC TGG -3' and 5'- ATT CCA GTA TTA CAT TTC CCC TCC CTC CCC -3', and rTth DNA Polymerase XL (The Perkin-Elmer Corporation) yielded a single amplified product of the expected size (4.8 kbp). The amplified DNA was cloned into pCRII-TOPO (Invitrogen Corporation) and sequence-verified. Metaphase chromosomes prepared from rat bone marrow cells were hybridized with the biotin-labeled DNA probes, and hybridized loci were visualized with fluorescein isothiocyanate–conjugated streptavidin. The chromosomes were counter-stained with 4',6-diamidino-2-phenylindole, and observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

DNA Transfection

A 1230-bp fragment carrying the entire coding region of *cyr61* cDNA and C-terminal six-times repeated histidine sequence was amplified by RT-PCR using rat aorta poly(A)$^+$ RNA as a template and a set of primers, 5'-AGG GAT CCG GCT TTT GTT GGT TCT GTG TCG C-3' and 5'-TCA GAT CTT TAT TAG TGA TGG TGA TGA TGA TGG TCC CTG AAC TTG TGG ATA TCG-3', corresponding to nucleotides 1 to 18 and 1166 to 1187 of the rat *cyr61* cDNA. The amplified DNA was inserted into the *Bam*HI/*Eco*RI cloning site of the

cytomegalovirus controlled mammalian expression vector pcDNA3 (Invitrogen Corporation) containing Neomycin resistance gene as a selection marker, and sequence-verified. The resultant plasmid was transfected into rat aortic cell line A7r5 (ATCC: CRL 1444) with Lipofectin (Invitrogen Corporation) according to the manufacturer's protocol. Stable transformants were selected in media containing G418 (Invitrogen Corporation), and designated as Cyr61-A7r5. pcDNA3 vector alone was used as control.

Western Blot Analysis

Conditioned media of Cyr61-A7r5 cells were applied to HiTrap Heparin (Amersham Biosciences Corporation) column at 4° C (1 ml column for 10 ml media). The column was washed with 10 mM sodium phosphate (pH 7.0), and bound proteins were subsequently eluted with increasing concentrations of NaCl (0.2 to 2.0 M) in the same buffer. The column fractions were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting. Western blots were probed with anti-penta His antibodies (Qiagen GmbH, Hilden, Germany) and stained with enhanced chemiluminescence detection reagents (Amersham Biosciences Corporation). Conditioned media of pcDNA3-transfected A7r5 cells were used as a control.

Assays for Cell Proliferation and DNA and Protein Syntheses

Cyr61-A7r5 and A7r5 control cells were cultured for various time periods, and the number of viable cells were counted using CellTiter-Glo Luminescent Assay System (Promega Corporation, Madison, Wisconsin) according to the manufacturer's protocol with a luminometer (MTP-700CL, Corona Electronic Company, Ibaraki, Japan). We confirmed that a serum-stimulated increase in cell number is associated with increased luminescence. DNA and protein syntheses were evaluated by the incorporation of $[^3H]$thymidine and $[^3H]$leucine (NEN Life Science Products, Inc.), respectively, into acid-insoluble materials essentially according to the previous description (Hatakeyama et al, 1994). Briefly, Cyr61-A7r5 cells were seeded in six well plates at a density of 1×10^5 cells/well, and grown for 2 days in Dulbecco's modified Eagle medium (Life Technologies, Inc.) supplemented with 10% FBS. Then the cells were pulsed either with $[^3H]$thymidine or $[^3H]$leucine for 12 hours at a final activity of 1 μCi/ml. After incubation, cells were fixed with ice-cold 10% (w/v) trichloroacetic acid, and the resultant acid-insoluble materials were processed for liquid scintillation counting.

Statistical Analysis

All experiments were performed at least three times. Data were combined and expressed as the mean ± SD. Statistical significance was determined using one-way ANOVA. A value of $p < 0.05$ was considered significant.

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