Abstract—CARP, a cardiac doxorubicin (adriamycin)-responsive protein, has been identified as a nuclear protein whose expression is downregulated in response to doxorubicin. In the present study, we tested the hypothesis that CARP serves as a reliable genetic marker of cardiac hypertrophy in vivo and in vitro. CARP expression was markedly increased in 3 distinct models of cardiac hypertrophy in rats: constriction of abdominal aorta, spontaneously hypertensive rats, and Dahl salt-sensitive rats. In addition, we found that CARP mRNA levels correlate very strongly with the brain natriuretic peptide mRNA levels in Dahl rats. Transient transfection assays into primary cultures of neonatal rat cardiac myocytes indicate that transcription from the CARP and brain natriuretic peptide promoters is stimulated by overexpression of p38 and Rac1, components of the stress-activated mitogen-activated protein kinase pathways. Mutation analysis and electrophoretic mobility shift assays indicated that the M-CAT element can serve as a binding site for nuclear factors, and this element is important for the induction of CARP promoter activity by p38 and Rac1. Thus, our data suggest that M-CAT element is responsible for the regulation of the CARP gene in response to the activation of stress-responsive mitogen-activated protein kinase pathways. Moreover, given that activation of these pathways is associated with cardiac hypertrophy, we propose that CARP represents a novel genetic marker of cardiac hypertrophy. (Hypertension. 2000;36:48-53.)

Key Words: doxorubicin ＊ proteins ＊ hypertrophy, cardiac ＊ brain ＊ natriuretic peptides ＊ protein kinases

In response to hemodynamic overload, several parallel and interconnected intracellular signal transduction cascades are activated and mediate their biological effects, which include cardiac hypertrophy. These intracellular pathways also activate transcription factors such as c-fos, c-jun, c-myc, and egr-1, which in turn regulate many genes involved in diverse cellular metabolisms, including myocardial growth and apoptosis. However, the induction of these genes in response to acute mechanical loading is transient, and the precise role of immediate-early response genes in the ongoing development of hypertrophy and progression to heart failure remains obscure.

Altered gene expression during a longer time course in response to cardiac hypertrophy or heart failure is characterized by the increase in the expression of the constitutive contractile proteins, natriuretic peptides (eg, atrial natriuretic peptide [ANP]), brain natriuretic peptide [BNP]), the growth factors, and their receptors, adrenergic receptors, and other receptors. Previous studies implicated the expression of ANP and BNP genes as a marker for ventricular dysfunction. Although the molecular mechanisms responsible for the upregulation of these peptides have not been fully understood, nuclear factors whose expression levels are closely associated with the cardiac function may be candidates for regulatory molecules involved in such a process.

By using the differential display methods of mRNAs expressed at distinct levels between control and doxorubicin-treated cardiac myocytes, Jeyaseelan et al identified CARP as a cardiac doxorubicin (adriamycin)-responsive protein whose mRNA levels are markedly downregulated by doxorubicin. In situ hybridization in developing mouse embryo, they demonstrated that CARP mRNA is specifically expressed in the heart. Duced amino acid sequence of CARP cDNA revealed 4 repeats of ankyrin motif, which appears to be involved in protein-protein interactions. In fact, Zou et al identified the same molecule by 2-hybrid screening, in which the authors used HF1a-binding protein YB-1 as bait. In the present study, we examined CARP expression during cardiac hypertrophy and tested the hypothesis that CARP can serves as a genetic marker of cardiac hypertrophy.

Methods

Animals

All procedures were approved by the Animal Care and Use Committee of Gunma University School of Medicine and were performed...
in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council. Male Wistar-Kyoto (WKY) rats (250 to 300 g, 15 to 16 weeks old; n = 7) and spontaneously hypertensive rats (SHR) (250 to 300g, 15 to 16 weeks old; n = 7; Imai) were housed under climate-controlled conditions. Pressure overload was produced with abdominal aortic constriction (sham-operated n = 6, pressure overloaded n = 18) as previously described. The Dahl salt-sensitive (DS) rats were supplied by Eizai Pharmaceutical Company. Male DS rats were fed a 0.3% NaCl (low-salt) diet after weaning until the age of 6 weeks, after which they were fed an 8% NaCl (high-salt) diet as previously describedd (7 rats at 6 weeks of age, 12 rats at 11 weeks of age, 8 rats at 18 weeks of age). Systolic blood pressure (SBP) was recorded in awake rats with tail-cuff sphygmomanometry (model UR5000; Ueda).

**RNA Extraction and Northern Blot Analysis**

Total RNA and Northern blot analyses were conducted as described previously. Radiolabeling of the probes, a 1-kb fragment of rat CARP cDNA sequence (courtesy of Dr. L. Kedes, Institute of Genetic Medicine, University of Southern California, Los Angeles, Calif.) and a 628-bp fragment of rat BNP cDNA sequence (courtesy of Dr. K. Kangawa, National Cardiovascular Center, Suita, Japan), was performed with a Boehringer-Mannheim random primer labeling kit.

**Plasmid Constructions**

Expression vector RSV/p38 (kindly provided by Jiahuai Han, The Scripps Research Institute, La Jolla, Calif.) and EKVrAcv12 (kindly provided by Michael Karin, University of California San Diego) have been described elsewhere. Human genomic clone that encodes CARP was isolated by screening the human leukocyte genomic library (HL1006d; Clontech) with the 3' labeled rat CARP cDNA. For the generation of luciferase reporter genes, the forward primers with a consensus motif underlined and mutations of wild-type sequence in the promoterless luciferase reporter gene vector pGL3 (Promega). The Parker cDNA sequence (courtesy of Dr. L. Kedes, Institute of Genetic Medicine, University of Southern California, Los Angeles, Calif.) and a 628-bp fragment of rat CARP cDNA sequence were isolated by screening the human leukocyte genomic library (HL1006d; Clontech) with the 3' labeled rat CARP cDNA. The forward primers with a consensus motif underlined and mutations of wild-type sequence in the promoterless luciferase reporter gene vector pGL3 (Promega).

For the generation of site-directed mutants of an M-CAT element in the CARP promoter, recombinant PCR with 2 rounds of amplification was performed. The PCR primers (mutations of wild-type sequence appear in bold) for M-CAT mutation were 5'-ACCAAAAGAAGCCGCTTC-3' (sense) and 5'-GAGGGCGCCCTTCTTGGT-3' (antisense). In brief, sense and antisense primers with the corresponding mutations were synthesized and incubated in separate reaction tubes with 5'-GCCGATCCTCAAGGTGAGC-3' as template. upstream primer (nucleotide -206), and reverse primer (nucleotide +170), thus yielding 2 subfragments that each contained the appropriate mutation. Subfragments were gel purified, and a second round of PCR was performed with upstream primer (nucleotide -206) and reverse primer (nucleotide +170). The PCR products were then isolated and subcloned into the KpnI/Xhol sites of plG3 as described. The resultant plasmid was designated as CARP-206(MCATmut)Luc. For the rat BNP promoter-luciferase reporter construct, which contains sequence from -1000 to +70 of the rat BNP gene, 2 oligonucleotide primers were designed based on the published sequence (Figure 1C): the 5'-primer with the KpnI site (underlined) was 5'-CCCCGGTACCCGTCTACACCTACTGAGTGAGGAAGA-3', and the 3'-primer with the Xhol site (underlined) was 5'-GGGCTCGAGGAGCTGGATGTGCTGCG-3'. The PCR was performed using the rat liver genomic DNA as a template. The PCR product was subcloned into the KpnI/Xhol sites of pGL3. All resultant plasmids were verified with sequencing.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts from neonatal rat cardiac myocytes were prepared as previously described. The sequences of the sense strand of double-stranded oligonucleotides used as probes or competitors in electrophoretic mobility shift assays (EMSA) were as follows, with a consensus motif underlined and mutations of wild-type sequence in bold: CARP(−42/+25), 5'-ACCAGAGGGATGCGCTTC-3'; CARP(−42/+25 mol/ω), 5'-ACCAGAGGGATGCGCTTC-3'; and BNP(−113/+95), 5'-CAGGAGGATGCGCTTCG-3'. Binding reactions were performed as previously described.

**Cell Culture and DNA Transfection and Luciferase Assay**

Neonatal rat ventricular myocytes were isolated from 1-day-old WKY rats as previously described. Cells were transfected with 1 µg reporter plasmid and 1 µg expression plasmid with Tfx-50 (Promega) according to the manufacturer’s procedure. After transfection, cultures were reFed with DMEM containing 10% fetal calf serum. Luciferase assays were performed as described previously. SB203580 was purchased from Calbiochem.

**Data Analysis**

Statistical analysis were performed by a Student’s t test with significant differences determined as P < 0.05. Correlation was performed with the use of simple regression analysis.

**Results**

**An Increase in CARP mRNA Levels During Cardiac Hypertrophy Induced by Acute or Chronic Pressure Overload**

To determine whether cardiac hypertrophy affects CARP mRNA levels, we performed Northern blot analysis and quantified the mRNA levels in the heart subjected to acute pressure overload by constraining the rat cardiac aorta. CARP mRNA levels were significantly increased by 2.7 ± 0.24-fold in the pressure-overloaded hearts (control versus pressure overload, P < 0.01) (Figures 1A and 1B). In agreement with the previous reports, BNP mRNA levels were markedly induced by pressure overload. We next performed Northern blot analysis of total RNA of the rat left ventricle at various developmental stages from fetus to adult to determine whether the CARP gene is considered to be a fetal gene. The results showed that CARP mRNA levels in the heart gradually increase during development (Figure 1C) in a manner similar to BNP mRNA levels. We further determined whether cardiac hypertrophy associated with chronic elevation of blood pressure affects CARP expression. At the age of 15 to 16 weeks, SHR showed a significant elevation in SBP compared with WKY rats (SHR versus WKY rats, P < 0.01) (Figure 1D). CARP mRNA levels were 1.6 times higher in SHR compared with WKY rats (WKY rats versus SHR, P < 0.01) (Figure 1E).

In DS rats, the most widely studied genetic model of salt-sensitive hypertension, supplemental dietary sodium increases blood pressure, but in the Dahl salt-resistant (DR) strain, supplemental dietary sodium has little effect on blood pressure. As shown in Figure 2A, the measurement of SBP of rats at 11 weeks of age showed that SBP of DS rats on a high-salt diet exceeded that of DS rats on a low-salt diet. CARP mRNA was more abundant in the H group than in the L group at 11 weeks of age (Figures 2B and 2C). SBP in the H group at 18 weeks, however, was comparable to that in the L group. Despite no significant difference in SBP at 18 weeks, the CARP mRNA level in the H group was significantly higher than that in the L group. Because of signs of
congestive heart failure as demonstrated by cardiac dilatation and pleural effusion (data not shown), as well as a significant increase in left ventricular weight–to–body weight ratio at 18 weeks in the H group compared with that in the L group (1.5-fold) (Figure 2D), an increased CARP expression may reflect not only elevated SBP but also cardiac hypertrophy and heart failure.

CARP mRNA Levels Are Correlated With BNP mRNA Levels in Dahl Rats

Because an increase in synthesis of BNP is closely associated with left ventricular dysfunction, we compared the BNP mRNA levels with CARP mRNA levels in the Dahl rat model that appears to represent the transition from compensated heart failure to decompensated heart failure. Data on CARP and BNP mRNA levels were available from the same heart in 47 rats (7 rats at 6 weeks of age, 12 rats at 11 weeks of age in the L group, 8 rats at 18 weeks of age in the L group, 12 rats at 11 weeks of age in the H group, 8 rats at 18 weeks of age in the H group). As shown in Figure 2E, CARP mRNA levels are significantly correlated with each BNP mRNA level.

Figure 1. CARP expression during cardiac hypertrophy and rat development. A, Changes in CARP and BNP expression in pressure-overloaded ventricles at 8 days after banding of descending aorta. B, Quantitative analysis of CARP levels in sham-operated (control) and pressure-overloaded rats. C, Changes in CARP and BNP expression during rat development. Total RNA was isolated from the left ventricles of fetal (F); 4-, 7-, 14-, and 28-day-old; and adult (A) rats. D, SBP for WKY rats and SHR at 15 to 16 weeks. E, CARP mRNA levels in SHR and WKY rat hearts. Total RNA was prepared from rat left ventricles. Bottom 18S rRNA for normalization. Values are mean±SEM.

Figure 2. CARP expression in DS rats. A, SBP at 6, 11, and 18 weeks in DS rats. L and H indicate DS rats fed a low-salt diet and a high-salt diet, respectively. B, Representative Northern blot of CARP mRNA. Bottom, 18S rRNA for normalization. C, Quantification of CARP mRNA levels in DS rats. D, Changes in left ventricular weight normalized for body weight (LV/BW). E, Positive correlation between CARP and BNP expression in DS rats. Each bar represents the mean±SEM. An arbitrary value of 1.0 was assigned to the 6-week-old rats.

Effects of p38 Mitogen-Activated Protein Kinase and Rac1 Activation on CARP and BNP Promoters

To determine the molecular mechanisms underlying the induction of CARP mRNA levels in pressure-overloaded cardiac hypertrophy and heart failure, we constructed the luciferase reporter plasmid CARP-1832Luc, which consists of 1832 bp of the 5′-flanking sequence and 170 bp of the 5′-untranslated region of the human CARP gene. This construct was then transiently transfected into primary cultures of neonatal rat cardiac myocytes along with p38 and the constitutive active form of Rac1 (V12Rac1) expression plasmid. Figure 3A shows that cotransfection with p38 or V12Rac1 increased luciferase activity driven from CARP promoter by 10.2- and 7.5-fold, respectively. Because of the apparent coordinate regulation between CARP and BNP mRNA levels, we assessed the effects of overexpression of p38 or V12Rac1 on the BNP promoter activity. Results were similar to that seen in CARP-1832Luc reporter gene; cotransfection of p38 or V12Rac1 expression plasmids increased luciferase activity driven from BNP promoter by 10.2- and 7.5-fold, respectively. Because of the apparent coordinate regulation between CARP and BNP mRNA levels, we assessed the effects of overexpression of p38 or V12Rac1 on the BNP promoter activity. Results were similar to that seen in CARP-1832Luc reporter gene; cotransfection of p38 or V12Rac1 strongly activated the BNP-1000Luc reporter gene, which contains sequence from −1000 to +70 of the rat BNP gene. Figure 3B shows that the ability of wild-type p38 expression vector to induce luciferase activity derived from CARP promoter is significantly attenuated in the presence of a specific inhibitor of p38, SB203580. These results confirm our conclusion that p38 activates CARP promoter.
M-CAT Box at −40 Mediates p38- and Rac1-Induced CARP Expression

To determine the cis-regulatory elements responsible for p38- or V12Rac1-induced CARP expression, a series of 5’-deletion constructs was transfected. Although removal of sequence from −1832 to −206 resulted in a ∼80% decline in basal reporter activity (data not shown), the fold-induction of promoter activity derived from CARP-206Luc in response to the expression of either p38 or V12Rac1 was comparable to that seen with CARP-1832Luc (see later). A search of the sequence downstream of −206 revealed the presence of a 5’-CATTCT-3’, or M-CAT, consensus sequence lying between −37 and −31 in the complementary strand. We then determined whether the M-CAT box in the CARP promoter could serve as a binding site for M-CAT box-binding protein or proteins. The incubation of nuclear extracts from cardiac myocytes with the radiolabeled double-stranded oligonucleotide containing M-CAT box gave rise to single protein-DNA complex (Figure 4A). Binding affinity of the nuclear proteins to M-CAT element in CARP promoter seems to be less pronounced than that to M-CAT element in the BNP promoter because 5 ng (10-fold molar excess) unlabeled BNP(−113/−95) completely competed for the binding to CARP(−42/−25), whereas 5 ng (10-fold molar excess) unlabeled CARP(−42/−25) only modestly competed for the binding to BNP(−113/−95) probe. As shown in Figures 4B and 4C, the point mutations, which abolished the interaction with M-CAT box-binding protein or proteins, resulted in a significant reduction in the responsiveness to the expression of p38 or V12Rac1. Thus, the M-CAT element in the CARP promoter is functionally important for p38- or V12Rac1-inducible activity of the CARP promoter.
Correlation Between CARP and BNP Expression
It should be noted that despite comparable SBP between L and H groups at 18 weeks of age, CARP expression was significantly elevated in the H group. These findings raised the possibility that factors other than the SBP can also contribute to the elevation of CARP mRNA levels. It has been demonstrated that the expression of proinflammatory cytokines such as interleukin-1β, monocyte chemotactic and activating factor, and monocyte chemoattractant protein-1 is increased in the DS rat hearts at 18 weeks of age. These findings led us to suggest that the augmented expression of CARP is in part ascribed to the increase in expression of these cytokines. Consistent with this hypothesis, we recently found that interleukin-1β increases CARP expression in vitro (data not shown).

Previous studies suggested that BNP levels are closely associated with the impairment of systolic function. The correlation of CARP with BNP mRNA levels in the Dahl rat model suggests that these 2 genes are regulated by shared mechanisms. Alternatively, transcription of the BNP gene may be regulated by CARP. The latter possibility, however, seems to be unlikely because the overexpression of CARP has little effect on BNP promoter as assessed with transient transfection assays and because adenovirus-encoding CARP had no effects on BNP mRNA levels in cardiac myocytes (data not shown).

Activation of CARP Promoter by p38 Mitogen-Activated Protein Kinase and Rac1 Through M-CAT Element
In an attempt to understand the mechanisms through which CARP expression is increased by cardiac overload, we investigated the roles of p38 and Rac1 in the CARP promoter activity because p38 and Rac1 have been implicated in hypertrophy of ventricular myocytes. Transcription factors such as c-Jun, ATF-2, and Elk-1 have been shown to be the major substrates of stress-responsive mitogen-activated protein (MAP) kinases, including p38. It has been demonstrated that phosphorylation of DNA binding domains by stress-responsive MAP kinases enhances DNA binding activity and activates transcriptional activity. However, the physiological consequence of the activation of stress-responsive MAP kinases largely remains controversial. Nemoto et al. indicated that p38 mediates hypertrophic agonist-induced ANP promoter, whereas JNK represses it. Our results with transient transfection and gel-shift assays indicate that p38 and Rac1 induce CARP promoter in an M-CAT element–dependent manner. M-CAT element has initially been described as an element that confers the muscle specificity to the cardiac troponin T gene. Subsequent studies have shown that the M-CAT element is critically involved in the inducible expression of several cardiac genes in response to protein kinase C or Ras activation. In this regard, our data expand understanding of the potential function of M-CAT element in mediation of the response to p38- and Rac1-dependent signals. Because a major form of M-CAT binding factor has been reported to be a transcription enhancer factor-1 (TEF-1), although there are multiple forms of the TEFs, it is intriguing to speculate that the transcriptional activating function of TEF-1 is regulated by phosphorylation via p38 MAP kinase cascade. Further studies are necessary to examine this possibility.

Model of Regulation of Cardiac Hypertrophy by CARP
Our observations suggest a model for CARP in the regulation of cardiac hypertrophy (Figure 5). The roles of stress-activated protein kinase (SAPK) in the development of the hypertrophic response have been studied extensively but are still far from conclusive. Previous studies to date have consistently demonstrated that hypertrophic agonists, including phenylephrine and endothelin-1, activate p38 in cardiac myocytes. However, the extent of the involvement of these pathways in the regulation of hypertrophic response remains controversial. The results of the present study implicated p38 in the transcriptional activation of CARP gene as well as the BNP gene through M-CAT–binding proteins. Because our recent experiments suggest that the overexpression of CARP by adenovirus inhibits protein synthesis and cellular enlargement, we assume that CARP exerts its inhibitory effects on cardiac hypertrophy. Taking into the consideration that ANP and BNP are highly inducible during cardiac hypertrophy and act as antihypertrophic peptides, the inducible expression of antihypertrophic protein CARP in response to pressure overload may be considered a fundamental mechanism that underlies the adaptation to cardiac overload.

In summary, we found that CARP expression is regulated by cardiac overload, including pressure overload, hypertension, and heart failure. The findings that M-CAT element mediates the induction of CARP and BNP promoters in response to stress-responsive MAP kinases will add to our understanding of how cellular stresses regulate these 2 genes. To the best of our knowledge, this is the first report that implicates a myocardial tissue–restricted nuclear factor as a genetic marker for cardiac hypertrophy.

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References


Cardiac Ankyrin Repeat Protein Is a Novel Marker of Cardiac Hypertrophy: Role of M-CAT Element Within the Promoter

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