Heart-directed Expression of a Human Cardiac Isoform of cAMP-Response Element Modulator in Transgenic Mice*

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The transcriptional activation mediated by cAMP-response element (CRE) and transcription factors of the CRE-binding protein (CREB)/CRE modulator (CREM) family represents an important mechanism of cAMP-dependent gene regulation possibly implicated in detrimental effects of chronic β-adrenergic stimulation in end-stage heart failure. We studied the cardiac role of CREM in transgenic mice with heart-directed expression of CREM-Ib C-X, a human cardiac CREM isoform. Transgenic mice displayed atrial enlargement with atrial and ventricular hypertrophy, developed atrial fibrillation, and died prematurely. In vivo hemodynamic assessment revealed increased contractility of transgenic left ventricles probably due to a selective up-regulation of SERCA2, the cardiac Ca2+ -ATPase of the sarcoplasmic reticulum. In transgenic ventricles, reduced phosphorylation of phospholamban and of the CREB receptor was increased, and messenger RNAs encoding transcription factor dHAND and small G-protein RhoB were decreased in transgenic hearts as compared with wild-type controls. Our results indicate that heart-directed expression of CREM-Ib C-X leads to complex cardiac alterations, suggesting CREM as a central regulator of cardiac morphology, function, and gene expression.

The transcriptional activation mediated by the cAMP-response element (CRE) and transcription factors of the CRE-binding protein (CREB)/CRE modulator (CREM) family represents an important mechanism of cAMP-responsive gene control (1). CREB and CREM bind as homo- or heterodimers to the CRE, a palindromic consensus element in gene promoters of numerous target genes. One mechanism of CRE-mediated transcriptional activation is the cAMP-dependent protein kinase A (PKA)-dependent phosphorylation of a critical serine in activating isoforms of CREM or CRE, finally leading to activation of the transcriptional complex (2). Inhibitory CREM or CRE isoforms lack functional domains that mediate transcriptional activation or regulation by phosphorylation. Those repressors bind to the CRE as homodimers or as heterodimers in combination with other activating or inhibitory isoforms and suppress transcriptional activation by displacing functionally active dimers from the CRE.

Several studies suggested that CRE-mediated transcriptional regulation plays an important role in cardiac gene regulation contributing to the pathophysiology of heart failure: (i) CREB and CREM are both expressed in human heart (3, 4); (ii) transgenic mice with heart-directed expression of a nonphosphorylatable, dominant-negative CRE isoform (dnCREB) (5) or of ATF3 (6), another repressor of CRE-mediated transcriptional activation, developed cardiac hypertrophy and signs of heart failure; and (iii) CREM-deficient mice (general knockout) displayed left ventricular dysfunction in the absence of hypertrophy and premature death (7, 8).

Here, we tested the role of CREM-Ib C-X, a CREM isoform previously isolated from human myocardium (4), in regulating cardiac function in vivo. This isoform belongs to a group of short CREB/CREM mRNAs, including CREB-W (9) and CREMΔC-X (10), which shows internal translation of small CREB/CREM proteins sharing a high degree of sequence homology: S-CREMα (13 kDa) and SS-CREMα (9 kDa) translated from CREMα C-X and other CREM isoforms (10), HIBI (11 kDa) and HIBII (9 kDa) translated from CREM-Ib C-X (4), and small inhibitory CREB proteins I-CREBI (16 kDa) and I-CREBS (8 kDa) translated from CREB-W (9). Those proteins are only composed of the respective basic region/leucine zipper domains for dimerization/CRE-specific DNA binding and act as suppressors of CRE-mediated transcriptional activation. Whereas CREB-W and CREMΔC-X are implicated in spermatogenesis and in decidualization of endometrial stromal cells, respectively (9, 10), the function of CREM-Ib C-X in the heart is not known.

We studied the specific cardiac role of CREM-Ib C-X in transgenic mice with heart-directed expression of this isoform fused to the hemagglutinin (HA) epitope to allow the immunological identification of CREM repressors HIBI and HIBII. We show that HIBII is generated from CREM-Ib C-X mRNA in transgenic hearts in vivo, combined with HIBI in one of two
founder lines. In CREM-IbΔC-X transgenic mice, ventricular hypertrophy was accompanied by increased left ventricular (LV) contractility; selective up-regulation of SERCA2, the cardiac Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum, and of the β\(_1\)-adrenergic receptor (β\(_1\)-AR); by decreased phosphorylation of phosphohambin (PLB) and of CREB, probably due to increased activity of serine-threonine protein phosphatase type 1 (PP1); and by premature death. Transcription factor dHAND and small G-protein RhoB were identified as potential cardiac target genes of CREM-IbΔC-X, which were down-regulated in transgenic ventricles. In addition, ventricular hearts developed considerable dilatation of atria followed by atrial fibrillation with rapid ventricular response. Our results identify CREM as a central transcriptional regulator of cardiomyocyte function and gene expression with unique properties fundamentally different from other suppressors of CRE-mediated transcriptional activation.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals**—The transgene construct was generated by subcloning 5′-C-X fragment encoding hemagglutinin epitope-tagged human CREM-IbΔC-X cDNA (4) into pBRE-2 plasmid (Clontech) using Nhel/NotI restriction enzyme sites. A 533-bp fragment encoding the transgene was excised from this intermediate construct using KpnI/XhoI restriction enzyme sites and cloned in KpnI/Sall-digested pMHC-poly(A) vector containing a 5.5-kb murine cardiac α-MHC gene promoter fragment. The pMHC-poly(A) vector was a kind gift of Dr. J. Robbins (Cincinnati, OH)(11). The identity and orientation of the insert were confirmed by DNA sequencing. Transgenic FVB/N mice were generated by the Transgene Core Facility, Interdisciplinary Center for Clinical Research, University of Münster, using a NruI-excised DNA fragment with the expression cassette. Two independently derived founder mice (Tg1 and Tg2) were identified on Southern blots and were continued on the same background or crossed with CD-1 mice for a separate set of experiments. Transgenic FVB mice were verified by DNA sequencing. Transgenic FVB/N mice were generated as published (5). Experimental Animals—The transgene construct was generated by subcloning a 516-bp fragment encoding hemagglutinin epitope-tagged human CREM-IbΔC-X cDNA (4) into pBRE-2 plasmid (Clontech) using Nhel/NotI restriction enzyme sites. A 533-bp fragment encoding the transgene was excised from this intermediate construct using KpnI/XhoI restriction enzyme sites and cloned in KpnI/Sall-digested pMHC-poly(A) vector containing a 5.5-kb murine cardiac α-MHC gene promoter fragment. The pMHC-poly(A) vector was a kind gift of Dr. J. Robbins (Cincinnati, OH)(11). The identity and orientation of the insert were confirmed by DNA sequencing. Transgenic FVB/N mice were generated by the Transgene Core Facility, Interdisciplinary Center for Clinical Research, University of Münster, using a NruI-excised DNA fragment with the expression cassette. Two independently derived founder mice (Tg1 and Tg2) were identified on Southern blots and were continued on the same background or crossed with CD-1 mice for a separate set of experiments. Transgenic FVB mice were verified by DNA sequencing. Transgenic FVB/N mice were generated as published (5). Experimental Animals—The transgene construct was generated by subcloning a 516-bp fragment encoding hemagglutinin epitope-tagged human CREM-IbΔC-X cDNA (4) into pBRE-2 plasmid (Clontech) using Nhel/NotI restriction enzyme sites. A 533-bp fragment encoding the transgene was excised from this intermediate construct using KpnI/XhoI restriction enzyme sites and cloned in KpnI/Sall-digested pMHC-poly(A) vector containing a 5.5-kb murine cardiac α-MHC gene promoter fragment. The pMHC-poly(A) vector was a kind gift of Dr. J. Robbins (Cincinnati, OH)(11). The identity and orientation of the insert were confirmed by DNA sequencing. Transgenic FVB/N mice were generated by the Transgene Core Facility, Interdisciplinary Center for Clinical Research, University of Münster, using a NruI-excised DNA fragment with the expression cassette. Two independently derived founder mice (Tg1 and Tg2) were identified on Southern blots and were continued on the same background or crossed with CD-1 mice for a separate set of experiments. Transgenic FVB mice were verified by DNA sequencing. Transgenic FVB/N mice were generated as published (5).

**Northern Blot Analysis**—Total ventricular RNA was prepared using the RNA Midiprep Kit (Quiagen, Hilden, Germany). 3 μg/lane of total RNA were separated by agarose gel electrophoresis, transferred to nylon membrane (GeneScreen, DuPont, MA), and hybridized with radiolabeled cDNA probes specific for ANP or GAPDH following the manufacturer’s specifications. The specificity of PCR products was controlled by melting curve analyses and by standard PCRs following the protocol of local animal welfare authorities.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared from three mouse ventricles per group as published (5). Gel shift assays were performed with 14 μg of nuclear protein per binding reaction. Radiolabeled DNA oligonucleotides containing a perfectly complementary probe were radiolabeled with [\(^{32P}\)]dATP using T4 polynucleotide kinase (MBI Fermentas, St. Leon-Rot, Germany). Values for mRNA levels were determined in duplicates or triplicates with the help of LightCycler software version 3.5, using appropriate calibration curves obtained with different amounts of control cDNAs as standards. The specificity of PCR products was controlled by melting curve analyses and by standard PCRs followed by agarose gel electrophoresis.

**Statistics**—Data were presented as mean ± S.E. or as box plot (quantitative real time PCR). Statistically significant differences were determined using Student’s unpaired t test, the log rank test (survival), or the nonparametric Mann-Whitney test (quantitative real time PCR) with p < 0.05 considered as significant.

**RESULTS**

**Transgenic Mice Expressing Human Cardiac CREM**—We obtained two independent lines of transgenic mice, Tg1 and Tg2, with insertion of several copies of the transgene for expression of HA-tagged human CREM-IbΔC-X under control of the heart-specific α-MHC promoter. Northern blot analysis of cardiac total RNA revealed abundant mRNA production by the transgene (data not shown). A HA-specific antibody recognized a 14-kDa protein in ventricular homogenates from Tg2 and, more weakly, from Tg1 hearts (Fig. 1A). Another weak protein band of lower migration velocity was detected by the HA-specific antibody in hearts from Tg2 but not in Tg1. This indicates internal translation of HA-tagged CREM repressor proteins HhBI (96 amino acids) and HhBII (78 amino acids), which both have identical properties in regard to CRE-specific DNA-binding and suppression of CRE-mediated transcription (4), from CREM-IbΔC-X mRNA in vivo. The predominance of the smaller CREM protein, putative HhBI (78 amino acids), agrees well with the context around the second AUG in exon H (for translation of HhBII), representing a perfect Kozak sequence for effective translation, whereas the first AUG (for translation of HhBI) does not fully comply with the Kozak rules (4, 15). Both protein bands were not detected in lung or liver tissue and were observed in atrial and right ventricular specimens at similar levels as in LV samples (not shown).

Gel shift assays were performed with nuclear extracts from wild-type and Tg1 hearts in order to test whether the trans
Fig. 1. Expression and CRE-specific DNA binding of HA epitope-tagged CREM repressors HIb/HiBI and expression and phosphorylation of CREB in hearts from CREM-ΔC-X transgenic mice (Tg1 and Tg2) and control littermates (W). A, expression of HIb/HiBI in transgenic LV cardiac homogenates (Tg1 and Tg2). A 14-kDa protein band was detected in Tg1 and Tg2, whereas a weak second band was only visible in Tg2 (upper arrow); there was no signal detectable in W homogenate. B, CRE-specific binding. Cardiac nuclear extracts were incubated with a radiolabeled CRE-containing DNA oligonucleotide derived from rat somatostatin gene promoter. A predominant gel shift (arrow 1) was present in W and Tg1 and was inhibited by a nonlabeled competitor DNA oligonucleotide (50-fold excess) containing a CRE derived from the human chorion gonadotropin α gene promoter (HGα-CRE, c) but not by a mutated HGα-CRE (m), indicating CRE-specific binding. A second CRE-specific shift (arrow 2) was observed in Tg1 and (weakly) in W and was supershifted by pre-incubation of nuclear extract with anti-HA antibody (*). C, immunoblots for total CREB (CB) and CREB phosphorylated at Ser113/133 (P-CB) in LV homogenates from Tg1 and W. Upper arrow reveals CRE-specific binding. Cardiac extracts from Tg1 and W were supershifted by pre-incubation with anti-HA antibody (*). Lower arrow denotes the phosphorylated form. D, statistical evaluation from six Tg1 (■) and six W (□). Note the decreased phosphorylated CREB/total CREB ratio in Tg1. *, p < 0.05 versus W.

Fig. 2. Pathological analysis and survival of CREM-ΔC-X transgenic mice (Tg1 and Tg2) and wild-type littermates. A, anatomy of hearts from Tg1 and Tg2 in comparison with W at an age of 12 weeks; longitudinal sections are shown in B. Note atrial dilatation and thrombi in transgenic hearts. C, histological analysis of left atria (LA) and ventricles (LV) from Tg1 and W; staining with hematoxylin and eosin; original magnification ×200. Note dilatation of the atrial wall, increased myocyte size (LV), and disturbed myocyte architecture in Tg1. D, Northern blot analysis of total RNA from Tg1 and W ventricles. Northern blot was hybridized with probes specific for ANP and GAPDH. Note the up-regulation of ANP in Tg1.
largement, increased heart weight, and induction of ANP) clearly indicate hypertrophy of Tg1 hearts. Mean survival times were decreased in both transgenic lines as compared with wild-type controls (Fig. 3). Occasionally, transgenic mice (Tg1) could be examined shortly after spontaneous death. Those hearts displayed the same morphological changes as shown for sacrificed mice (Fig. 2) (i.e. hypertrophy of atria and ventricles), and there were no signs of heart failure (namely ventricular dilatation, pleural effusion, or edema) visible in those mice. There were no significant differences in the proportion of transgenic and nontransgenic pups at the age of genotyping (3–4 weeks), indicating that mortality of young transgenic animals was not elevated (Tg1; data not shown). Since breeding of Tg2 mice was not successful over more than two generations, all experiments of this study were performed on Tg1 mice unless otherwise noted.

**Functional Assessment**—Atrial dilatation was accompanied by atrial fibrillation with rapid ventricular response in transgenic mice (Fig. 4A). Conversion to atrial fibrillation began at an age of 7 weeks, and atrial fibrillation was observed in all transgenic animals investigated at 16 weeks and older (n = 16). At 7 weeks, transgenic mice displayed sinus rhythm combined with enlarged atria (not shown), suggesting that atrial fibrillation occurred as a consequence of atrial dilatation. Cardiac function was studied in vivo by LV catheterization of Tg1 mice and littermate controls aged 12–14 weeks. Tg1 mice showed increased systolic LV function, as indicated by a significantly increased maximal rate of contraction (Fig. 4B and Table I). However, whereas all control mice displayed regular heart actions throughout the whole experiment, a significant portion of transgenic mice died during the experiment or were excluded from further analysis because of a highly variable, inconstant basal heart rate, probably due to atrial fibrillation. Therefore, assessment of LV function only extended to a selected group of Tg1 mice showing a regular heartbeat. In order to validate these data in a nonselected group of transgenic mice, we repeated catheterizations in CREM-Ib-X expressing mice and wild-type littermates with mixed genetic background generated by crossing-in CD-1 wild-type mice (Tg1FVB/N:CD-1, W) and P-waves, indicating sinus rhythm in W (arrows). B, hemodynamic analysis. Representative tracings of LV pressure (left axis) and first derivative of LVP (dP/dt, right axis) from Tg1 and W mice. Note the increased maximal rate of contraction in Tg1 as compared with W.

![Fig. 3. Kaplan-Meier survival analysis.](image)

**Fig. 4. In vivo assessment of cardiac function in CREM-IbΔC-X transgenic mice (Tg1) and wild-type littermates (W).** A, representative electrocardiogram tracings (leads I, II, and III) from Tg1 and W mice. Note atrial fibrillation with rapid ventricular response in Tg1 and W mice. Note atrial fibrillation with rapid ventricular response in Tg1 and W mice. B, hemodynamic analysis. Representative tracings of LV pressure (left axis) and first derivative of LVP (dP/dt, right axis) from Tg1 and W mice. Note the increased maximal rate of contraction in Tg1 as compared with W.

Different in the two groups, τ, the relaxation constant of LV pressure decay, was significantly shortened in transgenic mice from both genetic backgrounds as compared with the respective wild-type controls, indicating fastened LV relaxation in CREM-IbΔC-X transgenic hearts. Unequal basal hemodynamic parameters between W and W may be explained by previous observations that different mouse strains show considerable differences in left ventricular function. After stimulation with isoproterenol (40 pg/g body weight) maximal rates of contraction and of relaxation were increased, and time constant τ of LV relaxation was decreased in Tg1 as compared with W.

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**Table I**

Myocardial hypertrophy in CREM-IbΔC-X mice

|        | n  | BW | AW | VW | AW/BW | VW/BW |
|--------|----|----|----|----|-------|-------|
| W      | 23 | 26 ± 1 | 11 ± 1 | 109 ± 3 | 0.43 ± 0.02 | 4.13 ± 0.05 |
| Tg1    | 27 | 27 ± 1 | 78 ± 14* | 119 ± 4* | 2.99 ± 0.60* | 3.47 ± 0.10* |
| W      | 6  | 26 ± 1 | 13 ± 1 | 107 ± 5 | 0.50 ± 0.03 | 4.17 ± 0.12 |
| Tg2    | 4 (3) | 23 ± 1 | 76 ± 15* (n = 3) | 150 ± 7* | 3.40 ± 0.82* (n = 3) | 6.57 ± 0.21* |

* p < 0.05 versus W.
Transgenic Mice Expressing Human Cardiac CREM

Hemodynamic assessment of Tg1 and W mice (FVB/N) and of descendants of Tg1 with mixed genetic background (FVB/N:CD-1) is shown. LVP$_{\text{max}}$, maximal left ventricular pressure; dP/dt$_{\text{max}}$, maximal rate of contraction; dP/dt$_{\text{min}}$, maximal rate of relaxation; $r$, relaxation constant of LV pressure decay. In FVB/N:CD-1 mice, hemodynamic parameters were obtained in basal state (basal) and after injection of a maximal effective dose of isoproterenol (Iso; 40 pg/g). All Tg1 FVB/N mice displayed irregular heartbeats after infusion of isoproterenol.

| n  | HR | LVP$_{\text{max}}$ | dP/dt$_{\text{max}}$ | dP/dt$_{\text{min}}$ | $r$  |
|----|----|------------------|------------------|------------------|------|
|    | bpm | mm Hg | mm Hg/s | mm Hg/s | ms |
| FVB/N background |    |        |        |        |   |
| Basal | W 8 | 403 ± 11 | 93 ± 4 | 6773 ± 509 | -6260 ± 339 | 8.00 ± 0.27 |
| T 4 | 372 ± 38 | 102 ± 8 | 8122 ± 244$^a$ | -6688 ± 199 | 7.18 ± 0.13$^a$ |
| FVB/N:CD-1 background |    |        |        |        |   |
| Basal | W 5 | 372 ± 14 | 79 ± 4 | 3809 ± 366 | -3535 ± 344 | 7.00 ± 0.07 |
| T 6 | 407 ± 21 | 92 ± 3$^a$ | 6923 ± 580$^a$ | -6043 ± 546$^a$ | 6.15 ± 0.22$^a$ |
| Iso | W 5 | 531 ± 25 | 87 ± 4 | 7087 ± 812 | -4039 ± 346 | 6.06 ± 0.12 |
| T 6 | 536 ± 26 | 99 ± 4 | 10,465 ± 955$^a$ | -5834 ± 425$^a$ | 5.07 ± 0.04$^a$ |

$^a$ p < 0.05 versus W.

$^b$ p < 0.05 versus W.

Enhanced LV function in transgenic hearts under both basal and $\beta$-AR-stimulated conditions.

Expressional Changes in CREM-IbΔC-X Transgenic Hearts—Impaired cardiac contraction and relaxation in human failing hearts and in animal models of heart failure are associated with a down-regulation of $\beta_1$-AR, or of SERCA2, and with reduced phosphorylation of PLB at Ser16, a phosphorylation that inhibits SERCA2 activity, leading to an increased contractility during $\beta$-agonist stimulation (17–21). In order to test whether increased LV performance in CREM-IbΔC-X transgenic mice is accompanied by expression changes of these genes, we determined $\beta_1$-AR density and protein levels of SERCA2, as well as of calsequestrin, junctin, and phospholamban in ventricular homogenates. Protein levels of calsequestrin were determined as a negative control, since the expression of this protein is not changed in human heart failure and various cardiac animal models (17). $\beta_1$-AR density was increased by 10 fmol/mg protein or 32% in Tg1 as compared with wild-type controls (Table III). This increase was reflected by an increase in total ICYP binding by the same amount. SERCA2 protein was elevated to 127% in Tg1 ventricular homogenates, whereas expression of phospholamban, calsequestrin, and junctin remained unchanged (Fig. 5). A similar result was obtained in Tg1 FVB/N:CD-1 ventricles, showing a significant up-regulation of SERCA2 protein (PhosphoImager units; percentage of W$_{\text{FVB/N:CD-1}}$; SERCA2, Tg1 FVB/N:CD-1, 135 ± 8$^a$; W$_{\text{FVB/N:CD-1}}$, 100 ± 9 (n = 10); phospholamban, Tg1 FVB/N:CD-1, 100 ± 2; W$_{\text{FVB/N:CD-1}}, 100 ± 3$ (n = 5) calsequestrin, Tg1 FVB/N:CD-1, 107 ± 3; W$_{\text{FVB/N:CD-1}}, 100 ± 5$ (n = 9–10); $^a$ p < 0.05 versus W$_{\text{FVB/N:CD-1}}$).

In order to study the contribution of increased $\beta_1$-AR density/$\beta$-AR signaling to enhanced LV function in CREM-IbΔC-X transgenic mice, we determined the phosphorylation of PLB at Ser16 suggested to be the major mediator of $\beta_1$-AR mediated effects on SERCA2 function (22), as well as (CKII-dependent) phosphorylation at Thr17 in LV homogenates from Tg1 and W mice using phosphorylation-specific antibodies (Fig. 6, A and B). Phosphorylation of PLB at both sites was significantly reduced in Tg1 as compared with W. This indicates that increased PKA-dependent phosphorylation of PLB at Ser16 as a consequence of enhanced $\beta$-AR signaling is not a mechanism contributing to increased LV function in CREM-IbΔC-X transgenic mice. Decreased phosphorylation of PLB rather reflects a compensatory mechanism to increased LV performance, which inhibits SERCA2 activity. Furthermore, sustained $\beta_1$-AR signaling was reported to result in cardiac hypertrophy via activation of p38 MAP kinase and other MAP kinases (23, 24). Therefore, to study whether increased $\beta_1$-AR density/$\beta$-AR signaling contributes to cardiac hypertrophy via increased activation of MAP kinases in CREM-IbΔC-X transgenic mice, we...
CREM-Ib elements of activated forms of MAP kinases in cardiac ventricles from used in Fig. 5) and PLB phosphorylated at Ser16 (between phosphorylated and nonphosphorylated forms, same antibody as presented in PhosphorImager intensity units (W set to 100%). Note the decrease in p38 in T as compared with W; *, p < 0.05 versus W. C, immunological detection of activated forms of p38 MAP kinase (phosphorylated at Thr180 and Tyr182; p38), stress-activated protein kinase/c-Jun N-terminal kinase (phosphorylated at Thr183 and Tyr185; JNK), and p44/42 (Erk1/2) MAPK (phosphorylated at Thr202 and Tyr204; ERK) on Western blots with LV cardiac homogenates from T and W; representative autoradiographies. D, statistical evaluation from six T ( ) and six W ( ). Data are presented in PhosphorImager intensity units (W set to 100%). Note the decrease in p38 in T as compared with W; *, p < 0.05 versus W.

determined the activated (phosphorylated) forms of different MAP kinases using phosphorylation-specific antibodies in the same homogenates (Fig. 6, C and D). Whereas the phosphorylated form of p38 MAPK was significantly less abundant in Tg1 as compared with W, the signals for phospho-stress-activated protein kinase/c-Jun N-terminal kinase and phospho-p44/42 MAPK (phospho-Erk1/2) were not different in the two groups. Since cardiac hypertrophy combined with increased LV function was also reported in transgenic mice with heart-directed expression of Akt (protein kinase B) (25), we determined protein levels of the activated (phosphorylated) form of Akt using a phosphorylation-specific antibody. We did not observe any difference in phospho-Akt abundance between Tg1 and W homogenates, suggesting that activation of Akt is not implicated in the phenotype of this model (n = 6; data not shown).

Since phosphorylation of CREB and of PLB was reduced in CREM-transgenic hearts, we also studied the expression of the catalytic subunit of PKA and of CKII, which both were reported to phosphorylate Ser15/18/23 of CREB (2) as well as Ser10 and Thr17 of PLB, respectively (26). However, protein levels of PKA and of CKII were identical between groups (Fig. 5). Since activity of serine-threonine PP1 was reported to be altered in CREM-deficient mice (8), we examined whether PP1 and serine-threonine PP2A were differentially activated in CREM-transgenic hearts. PP1 activity was increased to 140% in Tg1 ventricles as compared with wild-type littermates, reflected by a similar increase in total protein phosphatase activity (Fig. 7A); PP2A activity was not different in the two groups. Because protein levels of the catalytic subunits of PP1 and PP2A were not changed in CREM-transgenic ventricles (Fig. 7B), PP1 activity was elevated due to post-translational or other mechanisms rather than increased levels.

In order to identify potential cardiac target genes of CREM, the mRNA levels encoding transcription factor dHAND (27) and small GTP-binding protein RhoB (28) (genes differentially expressed in Tg1 and wild-type myocardium as assessed by array hybridizations (not shown)) and the mRNA encoding the housekeeping gene GAPDH were determined by real time PCR in total RNA from Tg1 and control ventricles (Fig. 8). Whereas GAPDH mRNA was not different in groups, both mRNAs encoding dHAND and RhoB were significantly reduced in Tg1 as compared with wild-type controls, suggesting both as potential cardiac target genes of HIbII.

**DISCUSSION**

Ectopic heart-directed expression of human cardiac CREM isoform CREM-IbΔC-X in transgenic mice evoked a complex phenotype with changes in cardiac function that are contrary to alterations observed in CREM-deficient mice and fundamentally different from phenotypes of related mouse models with heart-directed expression of other suppressors of CRE mediators of transcriptional activation. CREM-IbΔC-X transgenic hearts showed complex changes in the expression or function of various regulatory proteins (i.e. SERCA2, β-AR, ANP, dHAND, RhoB, PP1, CREB, PLB, and p38 MAP kinase), implicating CREM in various signaling pathways and suggesting CREM as a central transcriptional regulator of cardiac function. Moreover, results from this organ-specific transgenic model substantiate a specific role of CREM in the cardiomyocyte, extending the knowledge from general knock-out models of CREM.

**CREM Preserves LV Function, Increasing Expression of SERCA2 and β-AR—CREM-IbΔC-X transgenic mice displayed increased basal and isoproterenol-stimulated contractility (dP/dt max) and enhanced velocity of relaxation (dP/dt min),**
combined with a selective up-regulation of SERCA2 and β1-AR. These alterations are contrary to those in mice with general knock-out of the CREM gene (i.e. decreased basal contractility and delayed relaxation combined with down-regulation of SERCA2 and of β1-AR) (7). Thus, results from both CREM-IbΔC-X transgenic and CREM knock-out mice consistently support the hypothesis that one important cardiac role of CREM is to preserve LV function by maintaining expression of SERCA2 and β1-AR. Increased SERCA2 protein levels well explain enhanced LV function in CREM-IbΔC-X transgenic mice, since SERCA2 transgenic mice with the same increase in ventricular SERCA2 protein (to 150% of controls) also showed unchanged heart rate and comparable changes of contractility and relaxation (29) as observed in the present study. On the contrary, CREM-deficient mice displayed similar changes in basal LV contractility and relaxation (7) as heterozygous mice with a null mutation of SERCA2 and a comparable reduction of SERCA2 protein (to 65% of controls) (30). Phosphorylation of PLB at Ser16/Thr17 was reduced in CREM-IbΔC-X transgenic hearts, possibly reflecting a compensatory mechanism to increased SERCA2 protein levels. This suggests that decreased phosphorylation of PLB cannot fully compensate effects of increased SERCA2 protein levels on LV function in this model. This hypothesis is in line with results from heterozygous SERCA2 knock-out mice (30, 31) showing that, vice versa, increased phosphorylation of PLB at Ser16/Thr17 (to 200 and 210% of controls, respectively), even in combination with a down-regulation of PLB protein levels (to 65% of controls), cannot fully compensate for a 35% loss of SERCA2 protein in regard to decreased LV function. Unfortunately, it is not known whether phosphorylation of PLB is also compensatorily decreased in SERCA2 transgenic mice (29). Furthermore, a functional predominance of SERCA2 up-regulation over decreased PLB phosphorylation agrees with results from Brittsan et al. (32), suggesting that only 40% of SERCA2 pumps are functionally regulated by PLB in vivo. Increased β1-AR density in principle may also contribute to elevated LV function or cardiac hypertrophy in CREM-IbΔC-X transgenic mice; however, several results from this study suggest that the moderate increase in β1-AR density (132% of controls) does not play a major role in this phenotype. (i) Basal and β1-AR-stimulated heart rates were not altered in both CREM-IbΔC-X transgenic and CREM-deficient mice, as compared with their particular wild-type controls. (ii) Phosphorylation of PLB at Ser16/Thr17 and of CREB at Ser119/133, at least in part mediated by PKA, was decreased, not increased, in CREM-IbΔC-X transgenic hearts. (iii) There was no fibrosis in CREM-IbΔC-X transgenic hearts, and deceased CREM-IbΔC-X transgenic mice did not show signs of heart failure. Contrasting with this, transgenic mice with heart-directed overexpression of β1-AR (15-fold overexpression) display cardiac fibrosis and rapid transition to heart failure (33). However, comparison of both transgenic models is complicated by the different degree of β1-AR up-regulation/overexpression, and we cannot formally exclude the possibility that up-regulation of β1-AR contributes to changes in older CREM-IbΔC-X transgenic mice. (iv) Activation of MAP kinases was not increased in CREM-IbΔC-X transgenic mice as compared with wild-type controls, suggesting that elevated β1-AR density does not account for cardiac hypertrophy via increased MAPK signaling in this model. However, on the contrary, it is conceivable that decreased activation of p38 MAP kinase contributes to elevated LV function in CREM-IbΔC-X transgenic mice, since inhibition of endogenous p38 MAPK activity enhanced cell contractility in adult rat cardiomyocytes (23).

**Different Cardiac Functions of CREM, Dominant Negative CREB (dnCREB), and ATF3**—The ventricular phenotype of CREM-IbΔC-X transgenic mice is fundamentally different from the phenotypes of transgenic mice with cardiac-specific expression of other suppressors of CRE-mediated transcriptional activation (i.e. a nonphosphorylatable dominant negative CREB mutant (dnCREB) (5) or the stress-inducible CRE-binding transcription factor ATF3 (6)). Transgenic mice expressing dnCREB develop signs of heart failure with depressed LV function, hypertrophy of ventricles, and edema. Similarly, ventricular hypertrophy and reduced contractility were reported in ATF3-expressing mice. These results stand in clear contrast to the increased LV performance of CREM-IbΔC-X transgenic mice and indicate important functional differences due to distinct sets of target genes between CREM, CREB, and ATF3 in the heart. It is not known whether RhoB and dHAND, the most prominent down-regulated genes in CREM-IbΔC-X transgenic ventricles, are differentially expressed in dnCREB or ATF3 transgenic hearts. However, the selective up-regulation of SERCA2 protein in CREM-IbΔC-X transgenic ventricles contrasts with results reported from ATF3 mice (6) and might therefore contribute to the different phenotypes of the particular mouse models.

CREM-IbΔC-X transgenic mice developed atrial fibrillation, probably as a consequence of dilatation and hypertrophy of atria, and might therefore represent a useful genetic mouse model to study the pathophysiology of this kind of arrhythmia. Despite the differences in ventricular phenotypes, transgenic mice expressing dnCREB and ATF3 show similar atrial changes, namely atrial enlargement and hypertrophy, combined with conduction abnormalities in ATF3 transgenic mice (5, 6). The underlying mechanism leading to atrial enlargement in the different mouse models is not known. However, results from CREM-IbΔC-X transgenic mice suggest atrial hypertrophy as a primary event, since atrial enlargement cannot be explained as a consequence of congestion due to impaired LV function and since end-diastolic pressures in aorta and LV were not elevated (not shown).

**CREM Reduces Phosphorylation of CREB and PLB. Increasing PP1 Activity—CREB expression was unchanged in CREM-IbΔC-X transgenic hearts, and gel shift assays revealed a similar pattern of CRE-binding proteins in cardiac nuclear extracts from both groups, except a strong complex produced by**
transgenic H1bII. Furthermore, the abundance of mRNAs encoding CREB/CREM-related AP-1 transcription factors c-Jun, JunB, Fra-1, Fra-2, c-Fos, JunD, and FosB was not different in the two groups (Tg1FVB/N, 12–16 weeks age, n = 5–7) as assessed by RNase protection assays (not shown). Thus, cardiac expression of CREM-IbΔC-X did not induce major expression changes in important related transcription factors. Surprisingly, phosphorylation of CREB was reduced, not compensatorily increased, in CREM-IbΔC-X transgenic hearts, which suggests decreased activation of cardiac CREB, further enhancing suppression of CRE-mediated transcriptional activation by transgenic H1bII. In this context, it should be noted that cardiac effects of decreased CREB phosphorylation are not known and that decreased phosphorylation of CREB may evoke other cardiac effects than heart-directed expression of dnCREB, a nonphosphorylatable dominant-negative mutant of CREB (5). Increased activity of PP1 well explains reduced phosphorylation of CREB in CREM-IbΔC-X transgenic ventricles, since PP1-mediated CREB dephosphorylation was identified as a major mechanism of attenuation of CRE-mediated transcriptional activation (34). Therefore, decreased CREB phosphorylation via increased PP1 activity in CREM-IbΔC-X transgenic hearts may represent a novel level of interaction between CREM and CREB. Interestingly, a similar increase of PP1 activity (by about 40%) was also reported in hearts from CREM-deficient mice (8); however, effects on cardiac CREB phosphorylation were not reported in this model. Increased PP1 activity also explains decreased phosphorylation of PLB at Ser16, since decreased PLB phosphorylation at Ser16 combined with unchanged phosphorylation at Thr17 was reported in transgenic mice with heart-directed overexpression of PP1 (35).

Transcription Factor dHAND and Small G Protein RhoB Are Potential Cardiac Target Genes of CREM—Up-regulation of both SERCA2 and β1-AR must be regarded as the final result of multiple changes in gene expression and subsequent alterations in various signaling pathways in CREM-IbΔC-X transgenic hearts. It cannot be explained as a direct effect of heart-directed expression of H1bII, a suppressor of CRE-mediated transcriptional activation. We used recent array technology to search for potential direct cardiac target genes of H1bII whose expression is diminished in CREM-IbΔC-X transgenic ventricles and identified dHAND and RhoB as the genes most prominently down-regulated on the mRNA level. Transcription factors of the HAND family, basic helix-loop-helix proteins, are central regulators of cardiac gene expression and cardiac morphogenesis (27, 36–38). Whereas the mRNA encoding dHAND was down-regulated in CREM-IbΔC-X transgenic ventricles, the mRNA encoding dHAND-related factor eHAND was not changed (real time PCR; data not shown). During mouse heart embryonic development, dHAND and eHAND are expressed in a complementary fashion and are restricted to segments of the heart tube fated to form the right and left ventricles, respectively (36). The knock-out of the gene encoding dHAND in mouse embryos resulted in embryonic lethality at embryonic day 10.5 from heart failure (36). Recent studies on the cardiac function of dHAND show that expression of genes important during heart development (e.g. ANP or α-myosin heavy chain) is positively regulated by dHAND interacting with myocyte enhancer factor-2C (39–41). Interestingly, dHAND mRNA is strongly expressed in human heart (42), and eHAND (but not dHAND) is down-regulated in explanted human failing hearts with ischemic or dilated cardiomyopathies (43). The Ras-homologous (Rho) GTPases are involved in the regulation of a multitude of cellular processes, such as malignant transformation and genotoxic stress-induced signaling (28). RhoB mRNA expression was found in the developing and adult heart (28, 44) and was reported to be induced upon stress (28). Inhibition of Rho family small G proteins suppressed stretch-induced hypertrophy in rat cardiomyocytes, suggesting an important role in the regulation of cardiac growth (45). Taken together, the down-regulation of mRNAs encoding dHAND and RhoB in CREM-IbΔC-X transgenic ventricles indicates that CREM is implicated in important pathways of cardiac growth, and one may speculate (unless it is proven by promoter studies) that dHAND and RhoB represent direct cardiac target genes of H1bII.

Possible Role of CREM in the Pathophysiology of Heart Failure—Human heart failure develops as a consequence of a variety of cardiac diseases (e.g. ischemic or dilated cardiomyopathy), and failing myocardium is characterized by hypertrophy and impaired contraction and relaxation (17, 18). SERCA2 and β1-AR are down-regulated in the failing heart, explaining impairment of contractile function, delayed myocardial relaxation, and decreased potency and efficacy of β1-AR agonists in these patients (17). There is growing evidence that chronic stimulation of the β1-AR and subsequent activation of the cAMP-dependent signaling pathway by elevated plasma catecholamines play a central role in the development and progression of heart failure and in the altered gene regulation in failing heart (46). In line with this hypothesis, long term treatment with β1-AR antagonists improves prognosis and cardiac function in heart failure patients (47), whereas β1-AR agonists increase mortality in these patients (48). Hence, cAMP-dependent transcriptional control by CREB and CREM might contribute to altered cardiac gene regulation after chronic stimulation of the β1-AR in the failing heart, and several studies, including this one, suggest that CREM is implicated in this regulation: (i) different CREM proteins, including putative H1bII and CREMα/β, were immuno precipitated from human cardiac ventricular tissue homogenates, and CREM-IbΔC-X was isolated as an abundant CREM isoform from human heart (4); (ii) desensitization of CRE-mediated transcriptional activation was observed after chronic stimulation of the cAMP-dependent signaling pathway in primary cardiomyocytes (12); (iii) up-regulation of ICER, a CRE-responsive inhibitory CREM protein, has been described in neonatal rat cardiomyocytes upon β1-AR stimulation (49), well explaining desensitization of CRE-mediated transcriptional activation; and (iv) data from both CREM knock-out and CREM-IbΔC-X transgenic mice consistently showed that CREM positively regulates the expression of SERCA2 and β1-AR, genes differentially expressed in failing heart. The detailed knowledge of CREM-mediated transcriptional control and of specific downstream targets of CREM in the human heart will therefore be of great interest for understanding the pathophysiology of heart failure.

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