A novel corticotropin-releasing factor receptor splice variant exhibits dominant negative activity: a putative link to stress-induced heart disease

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ABSTRACT A growing body of experimental and clinical studies supports a strong association between psychological stress and cardiovascular disease. An important endogenous cardioprotective role in heart physiology has been attributed to corticotropin-releasing factor receptor type 2β (CRFR2β). Here, we report the isolation of cDNA from mouse (m) heart encoding a novel CRFR2β splice variant. Translation of this insertion variant (iv)-mCRFR2β isoform produces a 421-aa protein that includes a unique C-terminal cytoplasmic tail. Our functional analysis and cellular localization studies demonstrated that when coexpressed with wild-type mCRFR2β, iv-mCRFR2β significantly inhibited the wild-type mCRFR2β membrane expression and its functional signaling by ER-Golgi complex retention, suggesting a dose-dependent dominant negative effect. Interestingly, mice exposed to a 4-wk paradigm of chronic variable stress, a model of chronic psychological stress in humans, presented significantly lower levels of mCRFR2β and higher levels of iv-mCRFR2β mRNA expression in their hearts, compared to nonstressed control mice. The dominant-negative effect of iv-mCRFR2β and its up-regulation by psychological stress suggest a new form of regulation of the mCRFR2β cardioprotective effect and a potential role for this novel isoform in stress-induced heart disease.—Sztainberg, Y., Kuperman, Y., Issler, O., Gil, S., Vaughan, J., Rivier, J., Vale, W., Chen, A. A novel corticotropin-releasing factor receptor splice variant exhibits dominant negative activity: a putative link to stress-induced heart disease. FASEB J. 23, 2186–2196 (2009)

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Chronic cardiovascular diseases (CVDs) are the leading cause of death in developed countries. Exposure to chronic and acute psychological stress is an accepted risk factor for cardiovascular disease and may play a role in the development of cardiovascular pathology (1).

The hypothalamic peptide corticotropin-releasing factor (CRF) (2) plays a crucial role in the regulation of the hypothalamic-pituitary-adrenal axis and, collectively with its related peptides urocortin (Ucn) 1 (3), Ucn 2 (4, 5), and Ucn 3 (5, 6), acts to integrate the endocrine, autonomic, and behavioral response to stressors. The CRF-related peptides signal through the activation of two G protein-coupled receptors, CRF receptor type 1 (CRF1) (7–9), and CRF2 (10–14). Both CRF1 and CRF2 belong to the class B1 subfamily of seven-transmembrane domain receptors that signal by coupling to G proteins. CRF1 has only one known functional splice variant (α) expressed in the central nervous system and the periphery (15, 16); however, recently it has been demonstrated that in human skin, nonfunctional splice variants of CRF1 can interact and modify the CRF1α subcellular localization and thus may modify its activity (17, 18). CRF2 has three functional membrane splice variants in human (α, β, and γ) and two in rodent (α and β) (10–14, 16, 19). CRFR type 2α (CRF2α) is the major splice variant expressed in the rodent brain (20), whereas CRFR type 2β (CRF2β) is expressed primarily in peripheral tissues, with the highest levels of expression in the skeletal muscle, heart, and skin (10). CRF1 and CRF2 differ pharmacologically: CRF has relatively lower affinity for CRF2 compared to its affinity for CRF1. Ucn 1 has equal affinities for both receptors, and its action is mediated via CRF2β (for a review, see refs. 21–23). Exogenously administered Ucn 1 causes vasodilatation; increases heart rate, cardiac output, cardiac contractility and coronary blood flow; and has a positive ionotropic effect (24–26). These actions are...
absent in CRFR2-deficient mice (27, 28). In addition, Ucn 1, Ucn 2, and Ucn 3 protect against ischemic and reperfusion injury (29, 30) and have beneficial hemodynamic, hormonal, and renal effects in experimental heart failure models (31–33).

The expression levels of CRFR2β mRNA in the heart are down-regulated by different stressors, including exposure to LPS (34), subcutaneous ACTH or corticosterone injection (35), inflammatory cytokines (36), food deprivation (37), and acute or chronic stress (38).

Several natural mutations of GPCRs that have pathophysiological implications cause receptor retention within intracellular compartments [mostly in the endoplasmic reticulum (ER)]. These GPCR mutants often behave as dominant-negative forms by blocking surface expression of the wild-type receptor (39–42). In the this study, we present data on the isolation, characterization, and regulation of a cDNA from mouse heart encoding a novel CRFR2β splice variant with a dominant-negative activity.

MATERIALS AND METHODS

Isolation of the mouse insertion variant CRFR2β cDNA

The oligonucleotide primers 5’-TCGGGCAGGGGTAGGACAG-3’ (sense, exon 1, upstream to the ATG) and 5’-CGGCGAGGCTGACGAA-3’ (antisense, exon 14, downstream to the STOP codon) were designed to include the full mouse CRFR2 coding region. These primers were used for PCR amplification of cDNA prepared from mouse heart poly (A) RNA, which was reverse-transcribed using oligo(dT) primer. PCR was performed at 62°C for 35 cycles with 90s extension at 72°C. The amplified fragments were subcloned into pCRII-TOPO vector (Invitrogen Corp., Carlsbad, CA, USA), and individual colonies were digested using EcoRI to evaluate the inserted transcript size. A cDNA transcript of a larger size (127 bp) was observed, and sequence analysis found it to encode a novel splice variant bearing an insertion of an additional exon in intron 13.

Establishment of iv-mCRFR2β antisera

Antisera were raised in rabbits (PBL 6878, 6879, 6880), immunized with a synthetic peptide fragment encoding the unique C-terminal tail (aa 396-420: CQSSGPEFKPKTQ-TAEENLLNES) of ivCRFR2β conjugated to keyhole limpet hemocyanin via maleimide. Coupling protocol, formulation of immunogen for injection, and bleeding of host animals was as described previously in detail for inhibin subunits (43).

Animals

A total of 10 adult wild-type C57BL/6J male mice (Harlan, Jerusalem, Israel) were used for the experimental procedures. Mice were allowed 1 wk to acclimate before the beginning of experiments. Control mice (n=5) were housed in a temperature-controlled room (22 ±1°C) on a reverse 12:12 light-dark cycle. Food and water were given ad libitum. Chronic variable stress (CVS) mice (n=5) were housed in a temperature-controlled room (22 ±1°C) and were subjected to the CVS protocol for a period of 4 wk. In brief, mice were exposed to two different stressors per day, both in the dark and the light phase. The stressors were scheduled in two different weekly timetables, which were repeated twice to create unpredictability. The weekly stressors regime included unpredictable illuminations during the dark phase, male paired housing for 1 h, 30° cage tilt, damp bedding, low-intensity stroboscopic illumination at 180 strobes/min, 80 dB white noise, 15 min of swim stress in 28-cm-diameter tanks filled with 25°C water, 30 min of immobilization stress within a blunt 50-ml tube, and short periods of either food or water restriction, 4 h each. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Weizmann Institute of Science.

RNA preparation

Total RNA was extracted from mouse tissues using the TRizol RNA isolation reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer’s recommendations. The following mouse peripheral and CNS tissues were dissected and directly subjected to total RNA isolation: skin, skeletal muscle, heart, abdominal fat, and paraventricular nucleus of hypothalamus (PVN). Total RNA was also isolated from mice hearts following chronic variable stress procedure.

Semiquantitative and quantitative RT-PCR

Total RNA preparations derived from mouse tissues were reverse transcribed to generate cDNA pools. The cDNA products were used as templates for semiquantitative and quantitative RT-PCR analysis using specific primers for mCRFR2β, iv-mCRFR2β, the ribosomal protein S16, and hypoxanthine phosphoribosyl-transferase (HPRT). To avoid false-positive results caused by DNA contamination, we performed a deoxyribonuclease (DNase) treatment for 30 min at 37°C using the RNase-free DNase (Promega, Madison, WI, USA). We used semiquantitative RT-PCR to amplify the levels of endogenous mCRFR2β and iv-mCRFR2β present in the mouse tissues studied. The expression of ribosomal protein S16 served as an internal control. The PCR conditions were as follows: cDNA equivalent to 50 ng of total RNA was amplified by PCR for 40 cycles at an annealing temperature of 58°C. The final MgCl₂ concentration was 2 mM, and each reaction contained 0.5 U of Taq DNA polymerase (Hy Labs, Rehovot, Israel). We used quantitative RT-PCR to amplify the levels of mCRFR2β and iv-mCRFR2β present in mice hearts after chronic variable stress procedure. The expression of HPRT mRNA served as an internal control. The real-time RT-PCR reaction was performed in a Rotorgene 6000 thermocycler using fluorescent SYBR Green technology (ABgene, Epsom, UK). The PCR conditions were as follows: cDNA equivalent to 10 ng of total RNA was amplified by PCR for 45 cycles at an annealing temperature of 61°C. Each qPCR reaction contained 10 µl 2X SYBR Green Mastermix, and final primer concentration of 250 nM. The specificity of the amplification products was verified by melting curve analysis.

Oligonucleotide primers

Sense and antisense primers were selected, when possible, to be located on different exons to avoid false-positive results caused by DNA contamination. The following specific mCRFR2β, iv-mCRFR2β, and S16 oligonucleotide primers were used in the semiquantitative PCR reactions for mCRFR2β, 5’GGGGTTTACCCTTGTTGGGTAG3’ (sense) and 5’CTGAAAATTTCCACGTTGGTC3’ (antisense); for iv-mCRFR2β, 5’ATGACTGCCAAAGCTCAGGAC3’ (sense) and 5’ACAGGAGGTAT-GTGAGGTCA3’ (antisense); for S16,
HEK293T cells were plated at 75% confluence in 6-well plates precoated with poly-L-lysine. Cells were transfected using polyethylenimine (PEI) (Sigma-Aldrich, St. Louis, MO, USA) in a 1:1.5 ratio (DNA:PEI). The cells were washed and incubated in 1.8 ml serum-free DMEM (Invitrogen) per well. DNA mixture in total volume of 0.2 ml of serum-free DMEM was mixed by vortexing with 3 µl PEI (1 µg/µl), incubated for 10 min at RT, and the DNA/PEI complex was pipetted over each well. After overnight incubation, medium was replaced by growth medium (DMEM containing 10% FBS). HEK293T cells were used for the cyclic AMP-responsive element (CRE) activation studies. The cells were transfected with 1.5 µg of mCRFR2β or 1.5 µg of iv-mCRFR2β and 0.5 µg of a luciferase reporter containing a fragment of the EVX1 gene with a potent CRE response element site using the PEI method. The cells were washed with serum-free DMEM and then incubated for 4 h in serum-free DMEM containing DNA, 50 µg/ml chloroquine, and 33 µg/ml dextran. After incubation, the transfection mixture was discarded, and cells were incubated for 3 min in 660 µl serum-free DMEM containing 10% DMSO. Cells were then washed with serum-free DMEM, and medium was replaced by growth medium.

**Luciferase assay**

HEK293T cells were used for the cyclic AMP-responsive element (CRE) activation studies. The cells were transfected with 1.5 µg of mCRFR2β, iv-mCRFR2β or empty control vector. Twenty-four hours after transfection, cells were harvested, and the luciferase reporter activity was assayed as described previously (14).

**Immunohistochemistry**

For immunohistochemistry, paraffin-embedded sections of adult mouse hearts were used. Sections were stained with hematoxylin and prepared for avidin–biotin–immunoperoxidase localization of iv-mCRFR2β immunoreactivity using Vectastain Elite reagents (Vector Laboratories, Burlingame, CA, USA). Rabbit antiserum against iv-mCRFR2β (produced in the laboratory of W.V.) was used at a final dilution of 1:2000. Monoclonal antimyc antibody (Abcam, Cambridge, UK) was used at a final dilution of 1:2000. Antibodies against PD1 and P115, kindly provided by Dr. Zima Lev (Weizmann Institute, Rehovot, Israel) were used at a final dilution of 1:50 and 1:600, respectively. Hoechst 33342 (Invitrogen) was used at a final dilution of 1 µg/ml. G2 (Jackson ImmunoResearch, West Grove, PA, USA) and G3 (Chemicon-Millipore, Schwabbach, Germany) -conjugated secondary antibodies were used at a final dilution of 1:500.

**Surface expression analysis**

HEK293T cells were used for surface expression studies. The cells were transfected with 2 µg of mCRFR2β-myc or 2 µg of iv-mCRFR2β-myc for the surface expression analysis and cotransfected with 0.75 µg of mCRFR2β-myc and 0.0–0.75 µg of iv-mCRFR2β-myc for the dominant-negative analysis using the PEI method. Twenty-four hours after transfection, cells were harvested using trypsin and replated in a 96-well plate precoated with PLL. Forty hours after transfection, cells were fixed with fresh 4% PFA and stained with anti-myc antibody raised in mice (Abcam) at a final dilution of 1:3000, followed by horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Amersham Biosciences, Piscataway, NJ, USA) at a final dilution of 1:2000. 3,3′,5,5′-Tetramethylbenzidine (TMB) solution (Chemicon-Millipore) was used as substrate, and absorbance was measured in an ELISA reader (BioTek Instruments, Winooski, VT, USA).

**Analysis of protein levels**

HEK293T cells were transfected with 1.5 µg of mCRFR2β, iv-mCRFR2β or empty control vector. Twenty-four hours after transfection, cells were harvested in lysis buffer [25 mM Tris-HCl, pH 7.4; 150 mM KCl; 1.5 mM MgCl₂; 1% (w/v) glycerol; and 1% (w/v) Nonidet P-40] containing protease inhibitors. The samples were boiled for 5 min with sample buffer, and proteins were electrophoresed on 10% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA), transferred onto nitrocellulose membranes, probed with anti-iv-mCRFR2β fixed with fresh 4% PFA and stained with anti-myc antibody (produced in the laboratory of W.V.) was used at a final dilution of 1:5000. Monoclonal antimyc antibody (Abcam, Cambridge, UK) was used at a final dilution of 1:2000. Antibodies against PD1 and P115, kindly provided by Dr. Zima Lev (Weizmann Institute, Rehovot, Israel) were used at a final dilution of 1:50 and 1:600, respectively. Hoechst 33342 (Invitrogen) was used at a final dilution of 1 µg/ml. G2 (Jackson ImmunoResearch, West Grove, PA, USA) and G3 (Chemicon-Millipore, Schwabbach, Germany) -conjugated secondary antibodies were used at a final dilution of 1:500.

**Exonization prediction**

Prediction of acceptor and donor sites in the intron 13 of the human CRFR2 β gene was performed using the GeneQuest application of the Lasergene 7.2 software (DNASTAR, Inc., Madison, WI, USA).

**Statistical analysis**

All values are expressed as means ± s.e. Statistical analysis of these data was performed using Student’s t test. A level of $P < 0.05$ was accepted as statistically significant.

**RESULTS**

**Isolation of mouse CRFR2β insertion variant**

Oligonucleotide primers designed to include the full mouse CRFR2 coding region were used to amplify a
cDNA prepared from mouse heart poly (A) + RNA using PCR (Fig. 1A). The amplified fragments were subcloned, and individual colonies were digested to evaluate the inserted transcript size. A cDNA transcript of larger size (127 bp) was observed (Fig. 1C), and sequence analysis found it to encode a novel splice variant bearing an insertion of an additional exon in intron 13 (Fig. 1A, B). The new exon contains applicable acceptor and donor sites important for the regulated alternative splicing of this isoform (Fig. 1A). Translation of this insertion variant transcript (iv-CRFR2β) predicts a 421-aa protein, comprising the extracellular, intracellular, and transmembrane domains of CRFR2β but followed by a unique 35-aa C-terminal cytoplasmic tail (Fig. 1A, D). Screening of GenBank showed homology of the C terminus with no other protein. Bioinformatic analysis of the rat and human genome predicted the presence of a similar insertion variant in exon 13 of the rat CRFR2 gene, with a shorter cytoplasmic tail of 21 aa and homology of 70% in the amino acid sequence (Supplemental Fig. 1A). Exonization prediction of the human CRFR2 gene demonstrated a possible insertion of a short exon in intron 13 that will result in a unique 15-aa C-terminal cytoplasmic tail (Supplemental Fig. 1B). Experimental support for the presence of insertion variants in the CRFR2 gene of organisms other than mouse, which result in alternative cytoplasmic tail, should be further explored.

**Figure 1.** Sequence and schematic structure representation of iv-mCRFR2β. A) Schematic representation of the structure of the mCRFR2 gene, including the nucleotide and translated amino acid sequences of an exon inserted in intron 13. B) Schematic representation of the structure of the known mCRFR2β functional transcript (top) and the novel iv-mCRFR2β isoform. Locations of the translation start sites (ATG) are indicated for both transcripts. Exons coding for the N-terminal extracellular domain, the seven transmembrane domains, and the C-terminal cytoplasmic domain are indicated. Hatched boxes indicate 5’- and 3’-untranslated regions; open boxes represent coding regions. C) Representative image of electrophoretic analysis of the pCRII-TOPO vector containing the known mCRFR2β transcript (clones 1, 2, and 4) and the 127-bp-longer iv-mCRFR2β transcript (clone 3), isolated from a mouse heart. Oligonucleotide primers location used for iv-mCRFR2β isolation are indicated in A (P, primer; RP, reverse primer). D) Schematic representation of the amino acid sequence of the mCRFR2β and the iv-mCRFR2β, demonstrating a unique 35- vs. 46-aa intracellular tail for iv-mCRFR2β and mCRFR2β, respectively.
Expression of iv-mCRFR2 in mouse heart

To determine the tissue distribution of iv-mCRFR2β, a semiquantitative RT-PCR electrophoretic analysis was performed using mouse skeletal muscle, heart, and skin tissues that are known to express mCRFR2β (14) (Fig. 2A). Distinct from mCRFR2β mRNA expression, which is highly expressed by skeletal-muscle and heart tissue and to a lesser extent in skin (Fig. 2A, top), the iv-mCRFR2β mRNA was found to be restricted to the mouse heart (Fig. 2A, middle). To assess the iv-mCRFR2β protein expression, a highly specific antisemum was raised in rabbits using a synthetic peptide fragment encoding the unique C-terminal tail of iv-mCRFR2β (aa 396-420) (Fig. 2Ba). The specificity of the antibody was confirmed using both Western blot analysis (Fig. 2Bb) and immunofluorescence staining (Fig. 2C). The iv-mCRFR2β protein was only detected in cells transiently transfected with iv-mCRFR2β-expressing vector (Fig. 2Bb, lane 2; Ch). Cells transiently transfected with empty control vector (Fig. 2Bb, lane 1; Ca) or with mCRFR2β-myc-expressing vector (Fig. 2Bb, lane 3; Ce), did not demonstrate iv-mCRFR2β staining, confirming the specificity of the antibody. COS-M6 cells transfected with mCRFR2β-myc-expressing vector were positively stained using anti-myc antibody (Fig. 2Cd). The expression of the iv-mCRFR2β protein in the mouse heart was further demonstrated using immunohistochemical studies in mouse heart paraffin sections (Fig. 2E) and Western blots of mouse heart extract (Fig. 2D) using the anti-iv-mCRFR2β serum. Western immunoblot analysis of mouse heart extract reacted with anti-iv-mCRFR2β serum demonstrated a 45-kDa band (Fig. 2D), which corresponded to the size of the band detected in the iv-mCRFR2β-transfected cells (Fig. 2Bb). This band could not be observed in the skin extract (Fig. 2D). The cellular distribution of iv-mCRFR2β immunoreactivity in heart sections was widespread (Fig. 2Ea, c) and could not be demonstrated when heart sections were incubated with normal rabbit serum (Fig. 2Eb, d).

Cellular localization and function of iv-mCRFR2β

Binding of CRFR2 by the urocortin peptides initiates the accumulation of intracellular cAMP and activation of its subsequent signaling pathway (44, 45). To determine the functionality of the iv-mCRFR2β splice form, we assessed its ability to activate the cAMP pathway, in comparison to mCRFR2β, following ligand activation. To investigate the activation of the cAMP signaling in iv-mCRFR2β or mCRFR2β-expressing cells, we measured the activation of the cAMP response element (CRE) following treatment with different concentrations of Ucn 2 (Fig. 3A). HEK293T cells were cotransfected with a luciferase reporter construct containing a fragment of the EVX1 gene, which contains a potent CRE site, and the iv-mCRFR2β or mCRFR2β expression constructs. Cells were treated for 4 h with vehicle or with 0.01, 0.1, 1, 10, or 100 nM Ucn 2, and the luciferase reporter activity was measured and normalized on the basis of β-galactosidase activity (Fig. 3A). While Ucn 2 demonstrated a potent and dose-dependent activation of CRE

![Figure 2](image-url)
in mCRFR2β-expressing cells, it failed to stimulate the CRE in iv-mCRFR2β-expressing cells (Fig. 3A).

To further investigate the inability of iv-mCRFR2β to signal on stimulation with Ucn 2, we examined the surface expression and cellular localization of iv-mCRFR2β (Fig. 3B, C). A construct containing a myc tag at the first extracellular domain of iv-mCRFR2β was generated. COS-M6 cells transiently transfected with iv-mCRFR2β-myc-expressing plasmid were positively stained with antimyc antibody only following membrane permeabilization with Triton X-100 (Fig. 3Ba, b), demonstrating the absence of iv-mCRFR2β on the cell membrane. In contrast, COS-M6 cells transiently transfected with mCRFR2β-myc-expressing construct showed a strong membrane immunostaining using antimyc antibody, with or without membrane permeabilization (Fig. 3Bc, d). In addition, cell surface expression of iv-mCRFR2β and mCRFR2β was quantitatively evaluated using ELISA analysis (Fig. 3C). HEK293T cells transiently transfected with iv-mCRFR2β-myc-expressing construct showed minimal surface expression compared with cells transiently transfected with mCRFR2β-myc-expressing construct (Fig. 3C). As iv-mCRFR2β seemed to be retained intracellularly, we examined its internal localization (Fig. 3D). The iv-mCRFR2β protein expression was limited to the ER-Golgi complex, as determined by costaining of iv-mCRFR2β, transiently transfected into COS-M6 cells, and p115 (Fig. 3Da–c) or PDI (Fig. 3Dd–f) as Golgi or ER markers, respectively.

The dominant-negative effect of iv-mCRFR2β

Since both mCRFR2β and iv-mCRFR2β are endogenously coexpressed in the mouse heart, we examined possible functional interactions between these two splice variants by studying the effects of iv-mCRFR2β on mCRFR2β cAMP signaling. Transfected iv-mCRFR2β inhibits, in a dose-dependent manner, the cAMP re-
response to constant Ucn 2 (10 nM) treatment in HEK293T cells transfected with mCRFR2β, as measured by cAMP response element-luciferase activity of the EVX1 gene (Fig. 4A). Furthermore, the Ucn 2 dose-response activation of CRE-luciferase, mediated via mCRFR2β, was significantly inhibited by iv-mCRFR2β cotransfection, demonstrating 50 and 100% inhibition in 1:0.5 and 1:1 transfection ratios, respectively (Fig. 4B).

To further delineate the observed potent dominant negative effect of iv-mCRFR2β on mCRFR2β cellular signaling, we measured the surface expression (Fig. 4C) and cellular localization (Fig. 4D) of mCRFR2β in cells transfected with or without iv-mCRFR2β isoform. The surface expression analysis revealed a significant dose-dependent inhibition of mCRFR2β surface expression following iv-mCRFR2β coexpression (Fig. 4C). COS-M6 cells transfected with mCRFR2β-myc-expressing plasmid showed a clear membrane expression pattern (Fig. 4Da). In contrast, when cotransfected with iv-mCRFR2β, the mCRFR2β-myc was intracellularly retained together with iv-mCRFR2β (Fig. 4Db–d).

**Differential regulation of iv-mCRFR2β and mCRFR2β by stress**

To determine the effect of chronic stress on heart iv-mCRFR2β and mCRFR2β mRNA levels, we subjected mice to a chronic variable stress protocol for 4 wk. Control mice remained undisturbed beyond routine husbandry. The levels of mCRFR2β mRNA, determined using real-time PCR, were significantly reduced in mice subjected to chronic variable stress compared with control mice (Fig. 5A).

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**Figure 4.** Dominant-negative effects of the iv-mCRFR2β isoform. A) Activation of cAMP response element-luciferase reporter by Ucn 2 in HEK293T cells transiently cotransfected with a fixed amount (0.75 μg) of mCRFR2β construct, a luciferase reporter containing a fragment of the cAMP response element promoter of the EVX1 gene, and increasing amounts of iv-mCRFR2β construct (0.1–0.75 μg). Transfections were supplemented with empty pcDNA3 vector to control for total DNA transfected. Luciferase activity was measured 3 h after treatment with 10 nM Ucn 2. B) Activation of cAMP response element-luciferase reporter by Ucn 2 in HEK293T cells transiently cotransfected with 1:0, 1:0.5, and 1:1 ratios of mCRFR2β and iv-mCRFR2β, respectively. Luciferase reporter containing a fragment of the cAMP response element promoter was cotransfected into HEK293T cells with mCRFR2β and iv-mCRFR2β. Luciferase activity was measured 3 h after treatment with 0.001–100 nM Ucn 2. C) Surface expression of mCRFR2β measured by ELISA assay using monoclonal anti-myc antibody in HEK293T cells transiently transfected with 1:0, 1:0.5, and 1:1 ratios of mCRFR2β and iv-mCRFR2β, respectively. D) Immunofluorescence staining of COS-M6 cells transiently transfected with mCRFR2β-myc and stained with monoclonal anti-myc antibody followed by a Cy3-conjugated secondary antibody; note the full membrane expression of mCRFR2β (a). Immunofluorescence staining of COS-M6 cells transiently transfected with mCRFR2β-myc and stained with monoclonal anti-myc antibody followed by a Cy3-conjugated secondary antibody (b), and anti-iv-mCRFR2β serum followed by a Cy2-conjugated secondary antibody (c). Note the mCRFR2β retention in the ER-Golgi region and the colocalization with iv-mCRFR2β (merge and DAPI staining; d). Graphs shows mean of 8 replicates from one experiment.
The presence of a different cytoplasmic C-terminal tail in the iv-mCRFR2β splice form limited its ability to reach the plasma membrane of transfected HEK293T or COS-M6 cells. Although mCRFR2β protein showed a uniform plasma membrane pattern of cellular distribution, our cellular localization studies revealed that iv-mCRFR2β is concentrated around the nucleus and was colocalized with ER and Golgi markers, which corresponded to ER-Golgi retention. The intracellular retention of iv-mCRFR2β can be a result of the aa sequence modification in its C-terminal tail. In contrast to mCRFR2β that contains only one diarginine ER retention motif of the RXR type in its C-terminal tail, the iv-mCRFR2β C-terminal tail amino acid sequence includes a diarginine RRXRR ER retention motif, a dilysine KXK ER retention motif (49, 50), and an acidic dileucine EXXXLL motif known to mediate internalization targeting to endosomal-lysosomal compartments (51, 52).

The activation of mCRFR2β in distinct tissues or cell types by selective CRF and urocortin peptides initiates a variety of signaling pathways (for reviews, see refs. 53–55), which commonly involve an increase in cellular levels of cAMP. Our functional analysis studies demonstrated the inability of iv-mCRFR2β to induce cAMP/PKA signaling following Ucn 2 stimulation, probably due to its inability to reach the plasma membrane. Furthermore, coexpression of iv-mCRFR2β and mCRFR2β splice forms lead to a dose-dependent dominant-negative effect of iv-mCRFR2β on mCRFR2β-mediated cAMP pathway activation. Using cellular localization and surface expression analysis, we demonstrated that when cotransfected, iv-mCRFR2β impaired, in a dose-dependent manner, the mCRFR2β function by retaining its cellular location to the ER-Golgi complex. Several examples in the literature describe mutations in GPCRs that result in a truncated receptor retained in the ER with a similar dominant-negative effect (39–42). Two natural dominant-negative genetic mutations of the human chemokine receptor 5 (CCR5), an essential coreceptor for the cellular entry...
of the human immunodeficiency virus type 1 (HIV-1) (56, 57), were reported. The ccr5Δ32 (58) and the CCR5-893(−) (40) truncated forms of CCR5 were shown to be retained in the ER and inhibit the CCR5 surface expression by heterodimerization, conferring the cells’ resistance to HIV-1 infection. Another example exists in the Frizzled family of Wnt receptors. A mutant allele of Fz4, linked to the retinal degenerative disease familial exudative vitreoretinopathy, oligomerizes with wild-type Fz4, retains it in the ER, and inhibits its signaling (41). Recently, it has been demonstrated that coexpression of human skin CRFR1 splice variants with WT CRFR1 in epidermal keratinocytes resulted in intracellular retention of WT CRFR1. However, the functional importance of this intracellular retention was not reported (18). To our knowledge, iv-mCRFR2β is the only known isoform with a cellular dominant-negative effect at the CRF family.

Several studies have proposed an important endogenous cardioprotective role for CRFR2β in heart physiology (for a review, see refs. 21–23). The beneficial effects of urocortins, via activation of CRFR2β, include coronary vasodilatation, increased cardiac contractility, coronary blood flow and conductance, cardiac output, and heart rate, as well as protection against ischemia-reperfusion injury (24–26, 29, 30, 31–33). In addition, it was demonstrated that the expression of CRFR2β in the rodent cardiovascular system is down-regulated by different stressors (34–38). Systemic injection of LPS, a model of systemic immune activation and inflammation, markedly down-regulates CRFR2β mRNA levels in the heart in a dose-dependent manner (34). In the same vein, systemic administration of inflammatory cytokines like IL-1α and TNF-α also reduced the CRFR2β expression in heart (35, 36). Nutritional stress induced by food deprivation, acute physical restraint stress, and chronic isolation stress decreased CRFR2β mRNA expression in the atria ventricle, as well as the aorta of both female and male prairie voles (37, 38). In the present study, we showed, in concordance with the above previous studies, that the mCRFR2β mRNA levels were indeed down-regulated in hearts of mice that undergo chronic variable stress. In contrast, the iv-mCRFR2β mRNA levels were up-regulated, suggesting differential regulation by stress.

It has been recognized that psychological stress may be a trigger of CVD, including myocardial ischemia, myocardial infarction (MI), ventricular arrhythmia, and sudden cardiac death (for reviews, see refs. 1, 59, 60). The INTERHEART study (61) investigated the relation of chronic stressors to the incidence of MI in a sample of ~25,000 people from 52 countries. Those who reported “permanent stress” at work or at home had >2.1 times the risk for developing MI. The Whitehall II study (62) found 2.15-fold increased risk for new coronary heart disease in men who experienced a mismatch between effort and reward at work. However, downstream signals converting psychological stress into cellular dysfunc-

Several diseases were demonstrated to result from mislocalization of cell-surface GPCRs within cells. For example, retinitis pigmentosa is caused by a nonfunctional rhodopsin protein retained in the ER (63, 64), and nephrogenic diabetes insipidus is caused by intracellular retention of the vasopressin V2 receptor (64, 65). Furthermore, a recent study has demonstrated the association of abnormal C-terminal splicing variant in the cardiac sodium channel with human heart failure (66). The iv-mCRFR2 intracellular retention, the stress-induced change in the mCRFR2β to iv-mCRFR2β ratio, and the dominant-negative effect of iv-mCRFR2β on mCRFR2β, together suggest a possible mechanism for stress-induced heart diseases. Interestingly, we found high homology between the mouse and rat intron 13 at the insertion site of iv-mCRFR2β, and acceptor and donor sequences in the human intron 13, predicting the possibility of alternative splicing leading to exonization at the intron, which will result in the replacement of the C-terminal intracellular tail.

In conclusion, we found a novel heart mCRFR2β splice variant (iv-mCRFR2β) that acts as a dominant-negative regulator of mCRFR2β surface expression and which is up-regulated by chronic stress. The role of iv-mCRFR2β as a dominant-negative regulator of mCRFR2β under physiological and pathological conditions remains to be determined.

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