

# Characterization of a Novel Cardiac Isoform of the Cell Cycle-related Kinase That Is Regulated during Heart Failure<sup>\*[S]</sup>

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Myocardial infarction (MI) is often followed by heart failure (HF), but the mechanisms precipitating the transition to HF remain largely unknown. A genomic profile was performed in a monkey model of MI, from the myocardium adjacent to chronic (2-month) MI followed by 3 weeks of pacing to develop HF. The transcript of the gene encoding the cell cycle-related kinase (CCRK) was down-regulated by 50% in HF heart compared with control ( $p < 0.05$ ), which was confirmed by quantitative PCR. The CCRK sequence cloned from a heart library showed a conservation of the N-terminal kinase domain when compared with the “generic” isoform cloned previously but a different C-terminal half due to alternative splicing with frameshift. The homology of the cardiac sequence was 100% between mice and humans. Expression of the corresponding protein, measured upon generation of a monoclonal antibody, was limited to heart, liver, and kidney. Upon overexpression in cardiac myocytes, both isoforms promote cell growth and reduce apoptosis by chelerythrine ( $p < 0.05$  versus control). Using a yeast two-hybrid screening, we found an interaction of the generic but not the cardiac CCRK with cyclin H and casein kinase 2. In addition, only the generic CCRK phosphorylates the cyclin-dependent kinase 2, which was accompanied by a doubling of myocytes in the S and G<sub>2</sub> phases of the cell cycle ( $p < 0.05$  versus control). Therefore, the heart expresses a splice variant of CCRK, which promotes cardiac cell growth and survival; differs from the generic isoform in terms of protein-protein interactions, substrate specificity and regulation of the cell cycle; and is down-regulated significantly in HF.

Because the fully differentiated cardiac myocyte has a limited capacity for regeneration, most forms of heart disease, including ischemic heart disease and heart failure (HF),<sup>2</sup> are charac-

terized by a loss of cardiomyocytes (1–3). Therefore, deciphering the molecular mechanisms balancing cell growth and apoptosis may help develop strategies to improve the prognosis of both ischemic heart disease and HF, particularly with regard to stem cell therapy (4). Our previous studies have shown that genomic profiling in large mammalian models of heart disease allows the detection of novel potential candidates to promote cardiac cell growth and survival in a context of reversible ischemia or pressure overload (5, 6). In the present study, we extended this investigation to a model of postischemic cardiomyopathy in monkeys. We reasoned that a nonhuman primate model would be ideal, since it is phylogenetically and genetically closer to humans. Genomic profile by DNA microarrays in that model revealed the down-regulation of the cell cycle-related kinase (CCRK) after development of HF. Although this protein has not been characterized in the heart before, it is known that CCRK plays a major role in promoting the growth of cancer cells (7). Therefore, the goal of the present study is to characterize the expression of CCRK in the normal and failing heart and to determine whether this kinase is involved in cardiac mechanisms of cell growth and survival.

CCRK belongs to the family of cyclin-dependent protein kinase-activating kinases. A well characterized cyclin-dependent protein kinase-activating kinase is made of a complex that includes the catalytic subunit p40 MO15 (also called Cdk7 (cyclin-dependent kinase-7)), the regulatory subunit cyclin H, and the assembly factor MAT1 and activates the transcription factor IIH, which phosphorylates RNA polymerase II, as well as multiple components of the transcriptional machinery, including p53, RAR $\alpha$ , and Oct-1 (7–15). CCRK is distinct from MO15 and behaves differently in terms of size, substrate specificity, and immunological properties (16). CCRK is essential for the activation of the cyclin-dependent kinase Cdk2, a major regulator of the cell cycle (16). Cells lacking CCRK were incapable of growth, whereas cells with a reduced level of CCRK grew at a significantly slower rate than control cells (7, 16). Most interestingly, it was shown that a specific inhibition of CCRK promotes tumor cell death (7).

Our results demonstrate that the heart expresses a splice variant of CCRK, which differs from the “generic” isoform characterized in other tissues. The expression of the corresponding protein was confirmed upon generation of a monoclonal antibody. Overexpression of the cardiac CCRK in isolated cardiac myocytes promotes cardiac cell hypertrophy and survival. We also show that this cardiac variant differs from the generic isoform of CCRK in terms of developmental regulation, protein-

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1.

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<sup>2</sup> The abbreviations used are: HF, heart failure; CCRK, cell cycle-related kinase; MI, myocardial infarction; DAPI, 4',6-diamidino-2-phenylindole.

## Characterization of Cardiac CCRK

protein interactions, substrate specificity, and regulation of the cell cycle.

### EXPERIMENTAL PROCEDURES

**Monkey Model of HF**—Male monkeys (*Macaca fascicularis*, age 4–6 years, 3–8 kg) were used. The biological and physiological characteristics of these monkeys have been reported before (17, 18). Myocardial infarction (MI) was induced in five monkeys by ligation of the left anterior descending coronary artery. Instrumentation for measurement of left ventricular pressure and left ventricular dp/dt as well as for initiation of rapid right ventricular pacing was implanted. Eight weeks after infarction, monkeys underwent a 3-week protocol of pacing-induced (270 beats/min) HF. Sham monkeys ( $n = 5$ ) were used as controls (control group). The transition into HF was confirmed by measurements of ventricular pressure and contractility. Myocardial samples were taken from left ventricular areas adjacent to the MI and were immediately frozen in liquid nitrogen.

**Mouse Model of Ischemia/Reperfusion**—Experiments were performed on anesthetized mice under ventilation. After chest opening, ischemia/reperfusion was induced by occlusion of the left anterior descending artery for 30 min, followed by 24 h of reperfusion, as described previously (19).

**Oligonucleotide Microarrays**—Total RNA was extracted from about 300 mg of each monkey heart using the phenol/chloroform method (20). The concentration of RNA was measured spectrophotometrically by the absorbance at 260 nm. The procedure for RNA labeling was performed as described previously (19). Briefly, 15  $\mu$ g of mRNA were used for first strand cDNA synthesis with the SuperScript reverse transcriptase (Invitrogen) and a T7-oligo(dT)<sub>24</sub> primer. Second strand synthesis was performed with DNA polymerase I. Products were purified by Phase-Lock Gels/phenol-chloroform extraction. This DNA was subsequently transcribed into biotin-labeled synthetic antisense RNA, using the Bioarray RNA labeling kit (ENZO) with the T7 polymerase. Labeled cRNA was cleaned up (Qiagen), fragmented by alkaline treatment and hybridized to a GeneChip array overnight. Custom-made human genome chips (Center for Applied Genomics; University of Medicine and Dentistry of New Jersey) printed with a probe set representing 19,000 different genes were used to detect differences of gene expression. All values from each array were normalized to the 75th percentile value of the array, which was arbitrarily set at 100. Selected probe sets were analyzed by Student's *t* test. A *p* value less than 0.05 and -fold change greater than 1.2 (either up-regulated or down-regulated) were used to select probe sets whose intensity was considered significantly different between the two groups.

**Quantitative PCR**—Specific primers and probes (derived with FAM and TAMRA) were designed. For each measurement, the mRNA of interest was reverse-transcribed from 60 ng of total RNA with the TaqMan RT kit (Applied Biosystems). The gene-specific cDNA was used for quantitative PCR (40 cycles of a 10-s step at 95 °C and a 1-min step at 60 °C) using the TaqMan PCR mix (Applied Biosystems) on a 7700 ABI-Prizm sequence detector (Applied Biosystems). Standards were prepared for each transcript of interest from its PCR-amplified

cDNA after ligation of the T7 promoter (Ambion, Austin, TX) (21). Due to the variation in sample-to-sample loading conditions, the values of the transcripts were reported to the transcript level of cyclophilin, used as a housekeeping gene (17).

**CCRK Cloning and Sequencing**—The coding sequence of CCRK was amplified by PCR from both human and mouse heart cDNA libraries and subcloned into pGEMTeasy (Promega). PCR products were sequenced by triple pass on a 3100 genetic analyzer (Applied Biosystems) using the Big-Dye terminator (Applied Biosystems). Data analysis was performed with the ABI AutoAssembler software. Gene analysis and sequence comparisons were performed with the MacVector software.

**Generation of a Monoclonal Antibody against Cardiac CCRK**—Two epitopes matching the cardiac CCRK protein sequence were designed and synthesized. One epitope corresponds to the N terminus, which is shared by the generic CCRK and the cardiac variant. The second epitope recognizes only the C terminus of the cardiac variant. Using this strategy, we can measure both the total expression of CCRK (using the N-terminal antibody) and the expression of the cardiac specific splice variant (C-terminal antibody). The synthetic peptides were injected in BALB/c mice and tested by an enzyme-linked immunosorbent assay. Spleen of positive mice was harvested and fused with myeloma cells for production of a monoclonal antibody.

**Immunoblotting**—Tissues were homogenized at 4 °C in a lysis buffer (25 mmol/liter Tris-HCl, pH 8.0, 150 mmol/liter NaCl, 15 mmol/liter KCl, 1 mmol/liter EDTA, 1 mmol/liter dithiothreitol, 0.5% Triton X-100, and 5% glycerol), supplemented with protease, kinase, and phosphatase inhibitors, and then centrifuged at 12,000  $\times$  *g* for 20 min at 4 °C. Protein extracts (20–40  $\mu$ g) were denatured by boiling, resolved on SDS-polyacrylamide gels, and transferred to membranes. Primary antibodies were added at the recommended dilution and incubated overnight. After washing and incubation with the secondary antibody, detection was performed by chemiluminescence.

**Generation of a CCRK Adenovirus**—An adenovirus harboring either the coding sequence of the cardiac CCRK or of the generic CCRK was generated using the AdMax system (Clontech). The insert was subsequently digested and ligated downstream the cytomegalovirus promoter in a pCMV shuttle vector with loxP (pDC316) to tag the coding sequence with hemagglutinin at the N terminus (Clontech). The recombinant adenovirus was obtained in HEK293 cells by co-transfection of a cosmid (pBHGlox $\Delta$ E1,3Cre) containing the adenovirus type 5 genome (devoid of E1 and E3) with the shuttle vector containing the hemagglutinin-tagged CCRK sequence. The viral titer was determined on HEK293 cells overlaid with Dulbecco's modified Eagle's medium plus 5% equine serum and 0.5% agarose. Controls were performed with an adenovirus harboring the LacZ sequence.

**Cell Culture**—Ventricular cardiac myocytes were prepared from 1-day-old Wistar rats (Charles River Laboratories) (5), by digestion with 0.1% collagenase type IV (Sigma), 0.1% trypsin (Invitrogen), and 15  $\mu$ g/ml DNase I (Sigma) (22). Cell suspensions were applied on a discontinuous Percoll gradient (1.060/1.086 g/ml) made up in Ads buffer (116 mM NaCl, 20 mM

**TABLE 1**  
Preliminary data from the genomic profile

Name	Accession number	MI/HF versus control				
		A	B	C	D	E
G protein-coupled receptor 25	NM_005298	-3.43	-3.00	-3.92	-3.45	-4.43
KIAA0176 gene, partial cds	D79998	-3.13	-5.60	-2.59	-4.45	-4.16
Clone 24583 mRNA sequence	AF070595	-1.21	-2.11	-2.20	-3.27	-1.36
Rhodopsin homolog (RRH)	NM_006583	-2.68	-5.08	-4.50	-4.38	-4.52
Human BENE mRNA, partial cds	U17077	-1.66	-1.49	-3.49	-2.24	-1.04
CCRK	NM_012119	-1.59	-2.30	-1.45	-2.01	-2.34
6-Phosphogluconolactonase	NM_012088	-1.18	-2.72	-2.92	-2.70	-3.29

HEPES, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM MgSO<sub>4</sub>, pH 7.35) and spun at 1600 × *g* for 30 min. Myocytes were plated on gelatin-coated culture dishes at a density of 10<sup>6</sup> cells/cm<sup>2</sup> and cultured in modified Eagle's medium/F-12 supplemented with 5% horse serum, 4 g/ml transferrin, 0.7 ng/ml sodium selenite (Invitrogen), 2 g/liter bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 μM ascorbic acid, 100 μg/ml ampicillin, 5 μg/ml linoleic acid, and 100 μM 5-bromo-2'-deoxyuridine (Sigma). After 24 h, myocytes were cultured in serum-free conditions for 48 h before infection.

To measure cell survival, the infected myocytes were treated with 6 μM chelerythrine for 30 min or with 100 μM H<sub>2</sub>O<sub>2</sub> for 24 h, after which the cells were fixed in 10% formalin in phosphate-buffered saline for 10 min, washed in cold phosphate-buffered saline three times, and then incubated in 0.1% saponin in phosphate-buffered saline with 1 μmol/liter EGTA for 20 min at room temperature. DNA 3'-end labeling was performed using the biotin-16-dUTP/TdT system followed by fluorescein isothiocyanate-ExtrAvidin staining. The average number of myocyte nuclei per ×20 objective field was determined by manual counting of DAPI-stained nuclei using UV excitation. At the same magnification, terminal dUTP nick-end labeling-positive myocytes were counted.

To measure cell growth, infected cells were scraped with 1 ml of 1 × standard sodium citrate containing 0.25% SDS and vortexed extensively. The total cell protein and the DNA content were determined by the Lowry method and the Hoechst method, respectively. The protein content was normalized by the amount of DNA to correct for differences in cell number (23).

To determine the reentry into the cell cycle, infected cells were treated with 0.05% trypsin and centrifuged at 1000 rpm, and the pellet was resuspended with phosphate-buffered saline containing 20 μg/ml propidium iodide, 0.2% saponin, and 50 μg/ml RNase (Sigma), followed by incubation at room temperature for 30 min. The DNA content was analyzed by flow cytometry using a FACScan with fluorescence signals collected by a linear scale.

Myocyte nucleation was measured on formalin-fixed myocytes after DAPI staining and counterstaining of cardiac cells by α-actinin. Myocyte surface area was measured on these preparations with the Image-ProPlus Software System (Silver Springs, MD).

**Yeast Two-hybrid Assay**—Two constructs corresponding to the generic and the cardiac sequences of CCRK were designed as “baits” in a yeast two-hybrid system performed with the Matchmaker kit (Clontech) after ligation of each coding

sequence in Trp/pGBKT7-Myc vector and subsequent transformation in the AH109 yeast strain. Selection was performed on -Trp standard medium. This strain was mated with a Y189 strain pretransformed with a human heart library ligated in Leu/pGAD-hemagglutinin vector (Clontech). Co-transformants were grown on -His/-Leu/-Trp (low stringency) and -Ade/-His/-Leu/-Trp (high stringency) media (24). Positive clones were screened by sequencing. Positive interactions were verified upon co-transfection of plasmids harboring the Myc-tagged sequences of CCRK isoforms and a plasmid harboring the flagged sequence of the target protein.

**Statistical Analysis**—Results are presented as the mean ± S.E. We used Student's *t* test for two-group comparison. A value of *p* < 0.05 was considered as significant.

## RESULTS

**Characteristics of the Model**—A model of postischemic HF was developed in monkeys as detailed under “Experimental Procedures.” MI was induced in five monkeys by ligation of the left anterior descending coronary artery, and HF was induced by subsequent pacing. The HF group presented the typical functional characteristics of HF, including a hypertrophy of the myocardial tissue resulting from volume overload (left ventricle/body weight: 1.9 ± 0.2 versus 2.6 ± 0.5 g/kg in controls and HF, respectively; *p* < 0.05), an increased filling pressure (left ventricular end-diastolic pressure: 5.0 ± 0.5 versus 25 ± 5 mm Hg, respectively; *p* < 0.05), and decreased contractility as measured by the left ventricular +dP/dt (5,000 ± 2,000 versus 1,500 ± 500 mm Hg/sec, respectively; *p* < 0.05).

**Cloning of the Cardiac CCRK Transcript**—A custom-made DNA microarray showed that 1,259 genes were up-regulated in the HF group versus control, whereas 1,480 genes were down-regulated (supplemental Table 1). Table 1 summarizes the genes showing the highest amplitude of changes. Among these genes, CCRK (accession number NM\_012119) showed one of the most significant changes, with an average down-regulation of 2-fold in HF samples versus controls (Table 1). To verify that CCRK expression is down-regulated in the HF group, we designed a specific set of TaqMan probe and primers for quantitative PCR analysis, based on the human generic sequence available in public data bases. However, no PCR amplification could be detected with this assay. This negative result could be due to a difference in the CCRK sequence between humans and monkeys or due to the fact that the heart expresses a different transcript compared with the generic transcript reported in public data bases. To address that problem, we cloned the CCRK coding sequence from both human and mouse cardiac cDNA libraries. Both libraries showed that the CCRK sequence



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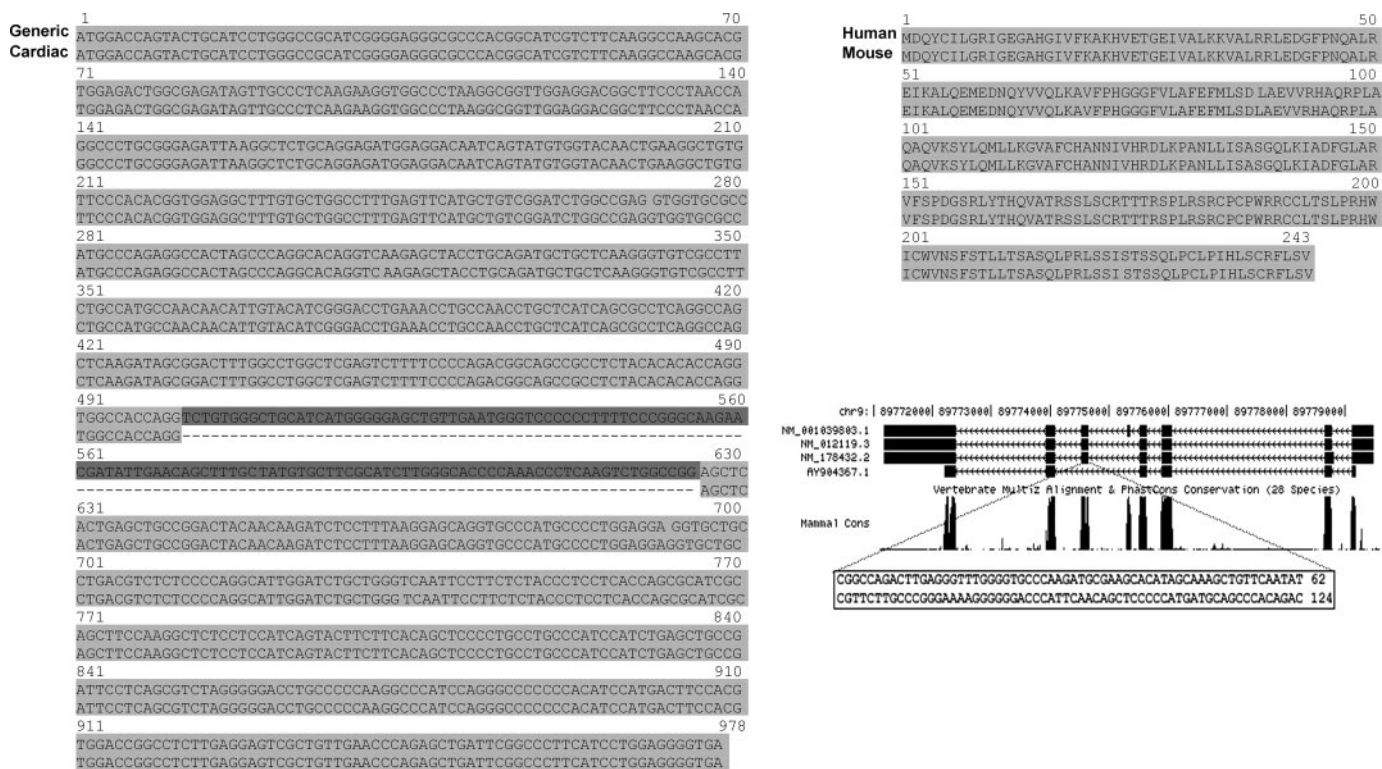


FIGURE 1. **Cloning of the cardiac CCRK transcript.** *a*, comparison of the cardiac CCRK sequence from a human heart cDNA library and the generic sequence. *b*, comparison of the human and mouse protein sequence in the heart. *c*, BLAT alignment of the cardiac and generic CCRK sequences, showing the exclusion of exon 3 in the cardiac sequence.

expressed in heart presents a deletion in the central part of the transcript and a frameshift in its C-terminal half when compared with the generic sequence reported in public data bases, whereas the N-terminal half, which contains the kinase domain, is strictly identical between both forms (Fig. 1*a*). Cloning of CCRK in other mouse tissues, including the liver, confirmed the presence of the generic sequence reported in GenBank<sup>TM</sup> (not shown). Remarkably, the protein sequence deduced from the cloning of the cardiac transcript expressed in both human and mouse libraries is 100% identical (Fig. 1*b*), which supports an important role for this variant across species. BLAT alignment of the cardiac transcript with the generic sequence shows that the cardiac isoform lacks exon 3, supporting the conclusion that this isoform results from alternative splicing (Fig. 1*c*). The coding sequences of the cardiac CCRK obtained from mouse, monkey, and human libraries were deposited in GenBank<sup>TM</sup> (accession numbers AY904369, AY904368, and AY904367, respectively).

The lack of amplification by quantitative PCR was explained by the fact that this PCR assay was targeting exon 3. A new quantitative PCR assay was therefore developed based on the sequence cloned from heart libraries. Using that assay, CCRK gene expression could be detected and showed a significant 2-fold decrease in the HF *versus* control (Fig. 2). Therefore, the down-regulation of this gene in HF found by microarrays is confirmed by quantitative PCR, both techniques showing a similar -fold change.

**Characterization of the Cardiac CCRK Protein**—Our next goal was to determine whether this alternative transcript of

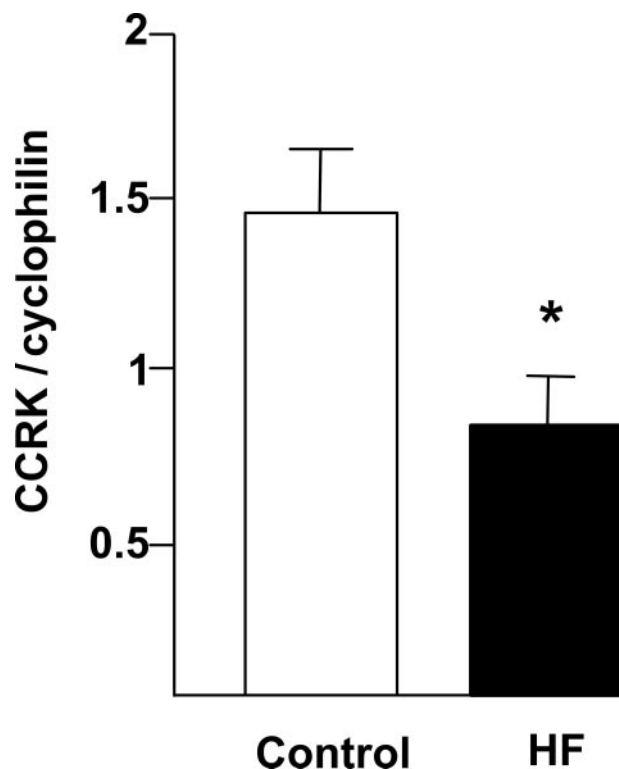
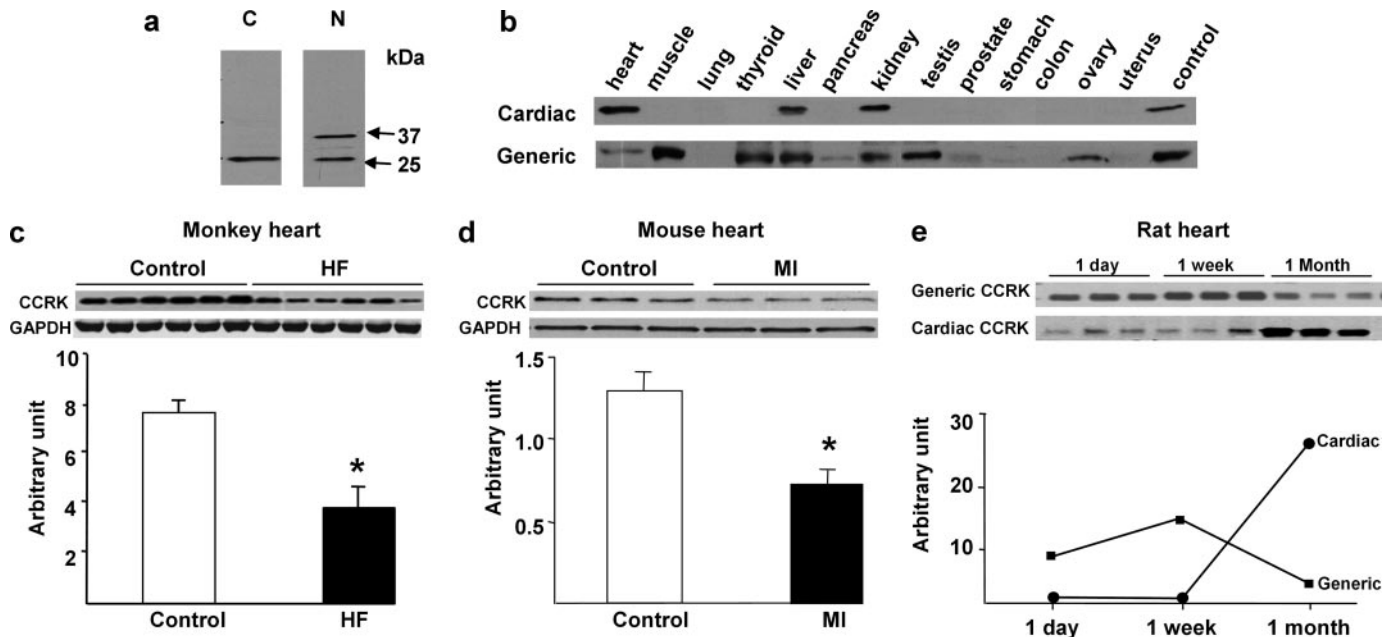


FIGURE 2. **Regulation of CCRK expression after HF.** Quantitative RT-PCR for the cardiac CCRK transcript measured in controls and HF monkey hearts ( $n = 4$  per group). \*,  $p < 0.05$  *versus* control.

CCRK corresponds to a native protein. A specific monoclonal antibody was generated, which targets the C-terminal half of the cardiac CCRK. Another monoclonal antibody was designed



**FIGURE 3. Characterization of the cardiac CCRK protein.** *a*, generation of monoclonal antibodies recognizing specifically the cardiac variant (C terminus) or both forms (N terminus) of CCRK. *b*, tissue distribution of both isoforms of CCRK in the monkey. *c*, decreased expression of the cardiac CCRK protein during HF in the monkey heart ( $n = 4$  per group).  $*$ ,  $p < 0.05$  versus control. *d*, decreased expression of the cardiac CCRK protein during ischemia/reperfusion in the mouse heart ( $n = 3$  per group).  $*$ ,  $p < 0.05$  versus control. *e*, developmental regulation of both CCRK isoforms in rat heart, comparing 1-day-old pups, and rats aged 1 week and 1 month.

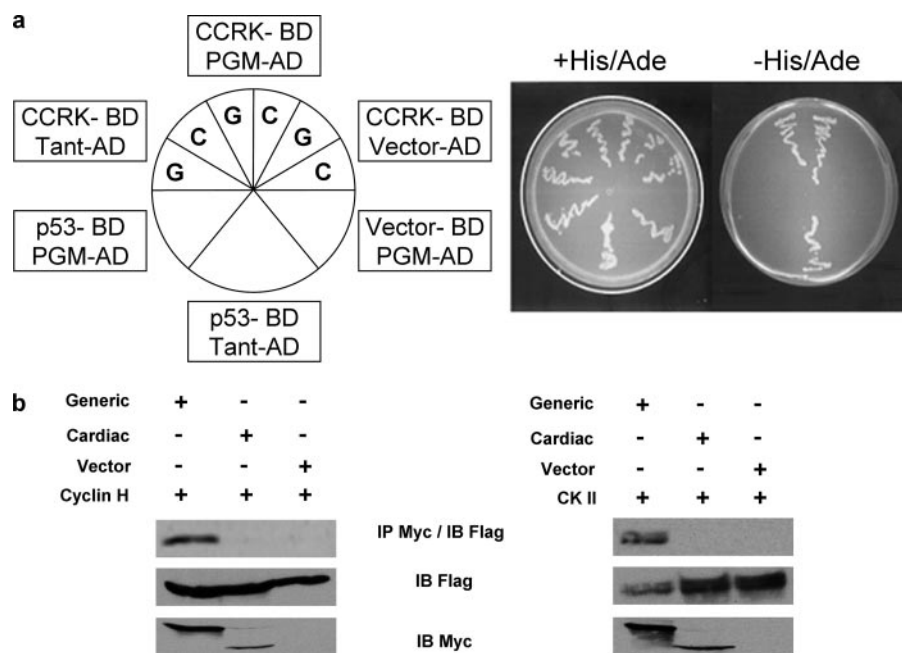
to recognize the N-terminal half of the molecule, which is identical between both isoforms. As shown in Fig. 3*a*, there was only one band detected at an approximate size of 25 kDa when using the C-terminal antibody, which corresponds to the expected size of the novel cardiac variant of CCRK found in the cloning experiment. Two major bands were detected when using the N-terminal antibody (Fig. 3*a*), at the approximate sizes of 25 and 37 kDa, which corresponds to the size of the novel cardiac variant of CCRK (25 kDa) and of the generic form of CCRK (37 kDa). Using the C-terminal antibody, we were able to confirm the expression of the novel CCRK protein in the monkey heart (Fig. 3*b*). This isoform could also be detected in liver and kidney (Fig. 3*b*). Interestingly, it was not found in skeletal muscle, a tissue that shows extensive homology with the heart in terms of protein profile. Reciprocally, the generic form of CCRK was detected in a larger array of tissues, including the heart, skeletal muscle, liver, kidney, testes, colon, and uterus (Fig. 3*b*). Using the C-terminal monoclonal antibody described above, we tested whether the protein corresponding to the cardiac isoform of CCRK shows the same regulation as the corresponding transcript during the transition into HF following MI. As shown in Fig. 3*c*, the expression of the cardiac CCRK protein was reduced by half in cardiac tissue samples from monkey subjected to HF when compared with control. Because we cloned the cardiac CCRK sequence from a mouse genome, we determined whether the protein would also be down-regulated in a mouse model of ischemia/reperfusion. Mice were submitted to 30 min of coronary artery occlusion followed by 24 h of reperfusion, after which CCRK expression was determined by Western blotting. As shown in Fig. 3*d*, the protein content was reduced by half ( $p < 0.05$ ) in the ischemic murine myocardium, reproducing the findings in the monkey model. Therefore, both

isoforms can be detected across species in the heart, and the cardiac CCRK shows a similar pattern of regulation after MI, both in rodents and in nonhuman primates.

We determined next whether both isoforms of CCRK are expressed concomitantly in the heart at different stages of development. Rat hearts were harvested from neonatal pups (1 day old) and from animals aged 1 week and 1 month. Expression of both isoforms of CCRK was measured by Western blotting with the monoclonal antibody recognizing the N terminus of the protein. Both proteins could be detected in the heart but at different time points (Fig. 3*e*). The expression of the generic CCRK was predominant in the neonatal heart, whereas it decreased markedly after 1 month, as cardiac myocytes matured (Fig. 3*e*). Remarkably, the cardiac isoform showed a reciprocal pattern of expression (*i.e.* a very low level of expression in the neonatal tissue and a predominant expression in the mature heart) (Fig. 3*e*).

**Protein-Protein Interactions**—We tested next whether the alternative splicing of the cardiac CCRK would modify the protein-protein interactions of the molecule. To answer that question, a yeast two-hybrid experiment was performed, using both the generic and the cardiac sequences as “baits” (Fig. 4*a*). Table 2 shows the potential interactions found for each isoform. Importantly, the clones obtained from the library mated with the generic CCRK showed a putative protein-protein interaction with casein kinase II and cyclin H, which was not found in the library mated with the cardiac CCRK (Table 2). These interactions were confirmed upon overexpression of the corresponding proteins in HEK 293 cells (Fig. 4*b*). The cells were transfected with an expression vector containing the flagged sequence of casein kinase II or cyclin H, together with an expression vector harboring the Myc-tagged sequence of either

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**FIGURE 4. Protein-protein interactions of CCRK.** *a*, yeast two-hybrid screening showing the different mating combinations plated on a culture dish with or without the restrictive agents (histidine and adenine). The T antigen/p53 mating is a positive control. PGM is the cardiac specific library. *b*, confirmation of the protein-protein interactions in HEK293 cells co-transfected with flagged cyclin H or CK II, together with a Myc-tagged cardiac or generic CCRK. An anti-Myc antibody was used for pull-down (IP), and the anti-FLAG antibody was used for immunoblotting (IB). Loading controls are shown by immunoblotting with FLAG and Myc. Vector, empty vector of pCS+6MT containing the cytomegalovirus promoter and six Myc tags at the N terminus.

**TABLE 2**

**Interaction of protein with generic and cardiac CCRK**

Generic CCRK		Cardiac CCRK	
Accession number	Name	Accession number	Name
NM_001320	Casein kinase 2	NM_012437	SNAP-associated protein
NM_001239	Cyclin H	NM_016081	Palladin
NM_173537	GTF2L repeat domain containing 1	NM_005502	ATP-binding cassette
NM_018216	Pantothenate kinase 4	NM_173193	Kv channel interacting protein 2 isoform 1

the generic or the cardiac isoform. As shown in Fig. 4*b*, there was a clear interaction between the generic CCRK and both casein kinase II and cyclin H, whereas such interaction was not found when co-transfecting the cardiac CCRK sequence.

**Substrate Specificity**—Because of these differences in protein-protein interaction, we determined whether the substrate specificity of cardiac CCRK would differ from the generic isoform. A well known substrate for the generic CCRK is Cdk2 (16). As shown in Fig. 5*a*, phosphorylation of Cdk2 on Thr-160 significantly increased after adeno-mediated overexpression of the generic CCRK in isolated cardiac myocytes, whereas such phosphorylation was not observed upon overexpression of the cardiac isoform. It is known that activation of Cdk2 controls the G<sub>1</sub>- to S-phase transition in cardiac cells (25). Therefore, we determined whether the overexpression of the generic isoform would affect the cell cycle in the cardiac myocytes. The phosphorylation of Cdk2 by the generic CCRK was accompanied by a significant increase in the percentage of myocytes in the S and G<sub>2</sub> phases of the cell cycle, as measured by flow cytometry (Fig. 5*b*). Reciprocally, overexpression of the cardiac CCRK did not affect the cell cycle (Fig. 5*b*). Accordingly, expression of

the proliferating cell nuclear antigen was increased upon overexpression of the generic but not the cardiac CCRK (Fig. 5*c*). Isolated cardiac myocytes infected with either isoform or with the LacZ control were stained by DAPI in order to determine whether the increased DNA synthesis by the generic isoform alters the ploidy or the nuclear count of the cardiac myocytes. As shown in Fig. 5*d*, myocytes infected with the generic but not with the cardiac isoform of CCRK showed up to a 4-fold increase in binuclear cells as compared with LacZ. These results demonstrate that both isoforms differ in terms of substrate specificity, cell cycle reentry, DNA synthesis, and myocyte nucleation.

**The Cardiac CCRK Promotes Cell Growth and Survival**—Because of this difference in substrates, further experiments were performed *in vitro* to better elucidate the function of the cardiac variant of CCRK and to determine its similarities and differences compared with the generic isoform. Neonatal rat cardiac myocytes were isolated and cultured. The expression of the cardiac CCRK protein was detected by Western blotting in the myocytes but not in the cardiac fibroblasts obtained from the preparation (Fig. 6*a*), which further demonstrates a myocyte-specific expression of the variant in cardiac tissue. Based on our hypothesis that the down-regulation of the cardiac CCRK during HF participates in the increased rate of cell death that characterizes that condition, we determined whether overexpression of the cardiac isoform of CCRK in cardiac myocytes would affect cell growth and survival.

The isolated myocytes were infected with an adenovirus harboring the sequence of either the cardiac or the generic isoform of CCRK and compared with a LacZ control. Cardiac cell growth was measured by the protein/DNA ratio. Infection with the adenovirus harboring the cardiac sequence significantly increased the protein/DNA content of cardiac myocytes by 36% at 48 h compared with infection with the same dose of the adenovirus harboring LacZ (Fig. 6*b*). Overexpression of the generic protein in cardiac myocytes showed a stimulation of cell growth that was quantitatively similar to the effect of the cardiac variant (Fig. 6*b*). However, we showed above that the generic CCRK increases DNA synthesis, which might affect the measurement of cell growth by protein/DNA ratio. Therefore, the surface of the myocytes was measured in the three groups. Although both isoforms of CCRK significantly increased cell surface as compared with LacZ, this increase was significantly more important in the group treated with the generic CCRK as compared with the cardiac isoform (Fig. 6*c*).

To examine whether the cardiac CCRK affects cell survival in cardiac myocytes, chelerythrine (6 μM) or H<sub>2</sub>O<sub>2</sub> (100 μM) was



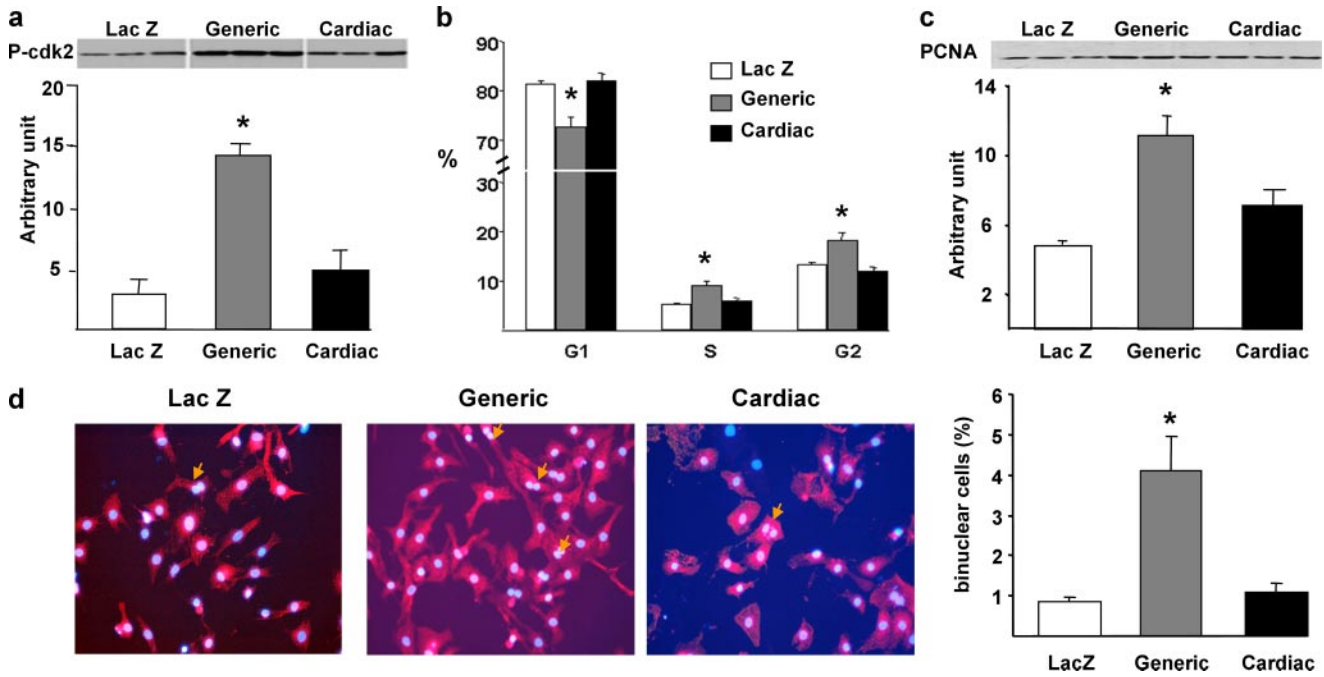


FIGURE 5. **Substrate specificity of CCRK.** *a*, phosphorylation of Cdk2 by the generic isoform but not by the cardiac isoform of CCRK. *b*, respective effect of both isoforms on the phase of the cell cycle, as measured by flow cytometry, in isolated cardiac myocytes. *c*, increased expression of proliferating cell nuclear antigen (PCNA) upon overexpression of the generic but not the cardiac CCRK isoform. *d*, measurement of myocyte nucleation after DAPI staining. The bar graph shows the percentage of binuclear cells in the different groups. \*,  $p < 0.05$  versus LacZ.

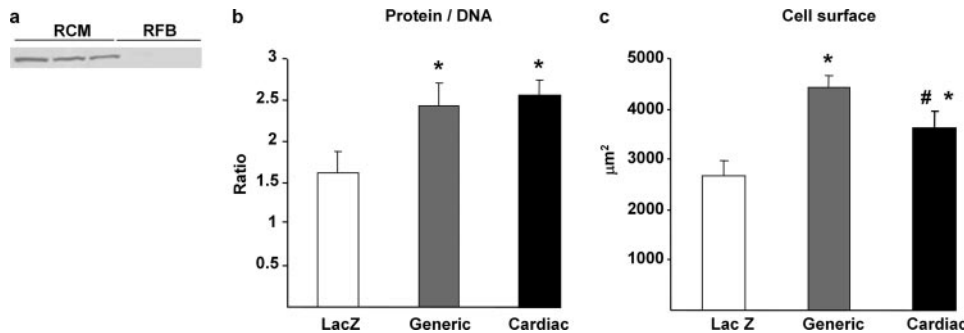


FIGURE 6. **CCRK promotes cardiac cell growth.** *a*, myocyte-specific expression of the cardiac CCRK. The protein can be detected by Western blotting in neonatal rat myocytes (RCM) but not in cardiac fibroblasts (RFB). *b*, effect of both the cardiac and the generic CCRK overexpression on cardiac hypertrophy, as measured by the protein/DNA ratio, in isolated cardiac myocytes, compared with LacZ controls. *c*, effect of both the cardiac and the generic CCRK overexpression on cardiac hypertrophy, as measured by cell surface area, in isolated cardiac myocytes, compared with LacZ controls. \*,  $p < 0.05$  versus LacZ; #,  $p < 0.05$  versus generic CCRK.

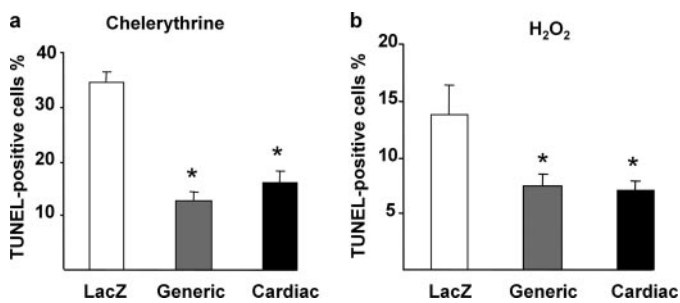


FIGURE 7. **CCRK promotes cardiac cell survival.** Apoptosis was stimulated in isolated cardiac myocytes upon the addition of chelerythrine (*a*) and H<sub>2</sub>O<sub>2</sub> (*b*). The effects of overexpression of both CCRK isoforms in terms of terminal dUTP nick-end labeling-positive cells are compared with the LacZ control. \*,  $p < 0.05$  versus LacZ.

added to the infected myocytes to induce apoptosis (Fig. 7). Cells infected with the cardiac CCRK showed a significant 50% reduction in apoptosis after the addition of chelerythrine (Fig.

7*a*) or H<sub>2</sub>O<sub>2</sub> (Fig. 7*b*) when compared with the LacZ control. Again, this protection was quantitatively reproduced by the generic isoform in both conditions (Fig. 7, *a* and *b*). We conclude from these experiments that both isoforms of CCRK promote cardiac cell growth and survival.

## DISCUSSION

The ischemic cardiomyopathy that follows MI induces an extensive remodeling of the ventricle, which includes increased myocyte cell death by both necrosis and apopto-

sis. We studied previously the genomic changes that participate in mechanisms promoting cardiac cell survival, both in the acute and chronic conditions of reversible myocardial ischemia (stunning and hibernation) (26–28), which led to the observation that more than 30% of the genes that are up-regulated in ischemic myocardium are involved in different mechanisms of cell survival, including resistance to apoptosis, cytoprotection (“stress response”), and cell growth (29). In the present study, we extended this genomic analysis to a monkey model of irreversible myocardial ischemia followed by HF.

Using a human DNA microarray in this model, we found that the cardiac expression of a gene encoding a novel isoform of CCRK is down-regulated after MI and even more after the transition into HF, which was confirmed by quantitative PCR. Specific monoclonal antibodies were generated, which show that the transcript of the cardiac CCRK encodes a native protein

## Characterization of Cardiac CCRK

following the same pattern of regulation during MI and HF. By cloning and sequencing the CCRK coding sequence from human and mouse heart libraries, we found that the CCRK transcript expressed in the heart is a splice variant of the transcript characterized in other tissues, including a frameshift that modifies the amino acid sequence of the C-terminal half of the molecule. Remarkably, the protein sequence deduced from the cloning of cardiac libraries in both humans and mice is absolutely identical, which supports an important role for this splice variant across species.

Whereas this study is the first to describe the cardiac variant, only few investigations have addressed the characterization and function of the generic CCRK. It has been shown that the generic CCRK is essential for Cdk2 activation through phosphorylation of Thr-160, which results in an activation of the cell cycle in cancer cells (16). Partial knockdown of the protein slows down the growth of HeLa cells, whereas complete silencing is incompatible with survival (16). Pharmacological inhibition of CCRK is cytotoxic for both cycling and noncycling cancer cells (7), which further highlights the importance of the prosurvival role of the molecule even independently from its effect on the cell cycle. In addition, knockdown of CCRK *in vivo* blocks the growth of glioma tumors (30).

Regulation of proteins involved in the control of the cardiac cell cycle is of direct therapeutic relevance. It has been shown that the expression of multiple activators of the cell cycle, including cyclins and Cdk2, increases after MI (31). Similarly, increased expression of cyclins reduces ischemic damage after MI, improves cardiac contractile function, and decreases the transition into HF (32–34). In that context, it could be argued that expression of the generic CCRK might be beneficial in the adult heart by promoting the cell cycling of the myocytes. However, a previous study has demonstrated that activation of Cdk2, which is the substrate of the generic CCRK (16), induces a maladaptive hypertrophic response to pressure overload in the adult heart (25). This observation further supports a biological function for the cardiac variant of CCRK, which retains the potential of promoting cardiac cell growth and survival without affecting Cdk2 activity. The observation that the generic isoform of CCRK induces maladaptive hypertrophy in the adult heart may explain the developmental regulation of both isoforms. The cardiac isoform shows a marked increase in expression level as the myocytes mature and exit the cell cycle, whereas the generic isoform shows a reciprocal pattern of regulation, which correlates with the observation that the generic but not the cardiac CCRK phosphorylates Cdk2 and promotes a reentry of the cardiac myocytes in the cell cycle. As such, the cardiac CCRK might function as a dominant negative isoform of CCRK in terms of cell cycle reentry in the adult heart; however, it maintains its role of promoting cell growth and survival.

A 37-kDa band was detected in multiple tissues, including liver, heart, muscle, and kidney, which matched the size of the generic mouse CCRK reported in public data bases. Reciprocally, a 25-kDa band was detected specifically in heart, liver, and kidney. Previous studies have shown that the expression of specific genes, especially those participating in energy metabolism and stress responses, can be restricted to the heart, liver, and kidney (35, 36). In addition, the cardiac CCRK presents several

interesting molecular characteristics that could lead to diagnostic or therapeutic applications. First, the cardiac CCRK is found in the heart but not in the skeletal muscle. This observation suggests an important role of the variant in the heart, because gene expression in heart and skeletal muscle are usually very similar. Because the cardiac form is a splice variant, its tissue specificity results probably from the expression of specific splicing proteins rather than transcription factors. It also leads to the possibility of using the cardiac CCRK as a marker for heart tissue because most of the proteins detected in plasma after MI (including lactate dehydrogenase or creatine kinase) can also be released from skeletal muscle. Second, our experiments confirm that both isoforms share the functional characteristics of promoting both cardiac cell growth and survival. This observation further supports the concept that the down-regulation of CCRK expression in a context of HF may impair cardiac cell survival. It also opens the possibility that a preemptive activation of survival pathways by acute CCRK overexpression might prevent cell loss in a context of myocardial ischemia.

In summary, our study characterizes a novel and tissue-restricted isoform of CCRK that participates in mechanisms of both cardiac cell growth and survival but acts as a dominant negative isoform in terms of cell cycle regulation in mature myocytes when compared with the more ubiquitously expressed generic isoform of the molecule. Besides its therapeutic and diagnostic potential, the down-regulation of cardiac CCRK expression after MI may be functionally involved in the transition of the ischemic myocardium into HF and in the increased rate of cardiac cell death that characterizes that condition. Further elucidation of the signaling mechanisms of CCRK in genetically modified animal models will lead to a better understanding of the pathways promoting cardiac cell growth and survival in the heart, which will also be critical to improving results for stem cell therapy.

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