Calreticulin signals upstream of calcineurin and MEF2C in a critical Ca\textsuperscript{2+}-dependent signaling cascade

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We uncovered a new pathway of interplay between calreticulin and myocyte-enhancer factor (MEF) 2C, a cardiac-specific transcription factor. We establish that calreticulin works upstream of calcineurin and MEF2C in a Ca\textsuperscript{2+}-dependent signal transduction cascade that links the endoplasmic reticulum and the nucleus during cardiac development. In the absence of calreticulin, translocation of MEF2C to the nucleus is compromised. This defect is reversed by calreticulin itself or by a constitutively active form of calcineurin. Furthermore, we show that expression of the calreticulin gene itself is regulated by MEF2C in vitro and in vivo and that, in turn, increased expression of calreticulin affects MEF2C transcriptional activity. The present findings provide a clear molecular explanation for the embryonic lethality observed in calreticulin-deficient mice and emphasize the importance of calreticulin in the early stages of cardiac development. Our study illustrates the existence of a positive feedback mechanism that ensures an adequate supply of releasable Ca\textsuperscript{2+} is maintained within the cell for activation of calcineurin and, subsequently, for proper functioning of MEF2C.

Introduction

The ER plays a key role in many cellular processes, including Ca\textsuperscript{2+} storage and release, protein synthesis, folding, and post-translational modification (Baumann and Walz, 2001). For example, Ca\textsuperscript{2+} release from the ER affects numerous cellular functions including modulation of apoptosis, stress responses, organogenesis, and transcriptional activity (Berridge et al., 2003). Calreticulin (CRT) is a Ca\textsuperscript{2+}-binding chaperone of the ER involved in Ca\textsuperscript{2+} storage and modulation of intracellular Ca\textsuperscript{2+} homeostasis (Michalak et al., 2002). Deletion of the CRT gene leads to embryonic lethality via impaired cardiac development, which results from malformation of the ventricular wall (Mesaeli et al., 1999). Immediately postpartum, the CRT gene is down-regulated because elevated expression of CRT in the fetal liver leads to the development of arrhythmias (bradycardia), sinus node depression, complete heart block, and, eventually, death from heart failure (Nakamura et al., 2001a). The expression of CRT is also altered in failing and hypertrophic hearts (Meyer et al., 1995; Hasenfuss et al., 1997; Tsutsui et al., 1997), indicating that this protein plays a role in postnatal/adult cardiac pathology. Cardiomyocytes are derived from the mesoderm and are produced in response to transcription factors, including bone morphogenetic proteins, which are secreted from adjacent endoderm (Srivastava and Olson, 2000). These signals activate numerous transcription factors (Nkx2.5, dHAND, eHAND, Sox-4, myocyte-enhancer factor [MEF] 2, nuclear factor of activated T-cells [NF-AT], and GATA), several of which may be Ca\textsuperscript{2+} dependent, and play a critical role in specific stages of vertebrate cardiac morphogenesis and hypertrophy (Srivastava and Olson, 2000; Chien and Olson, 2002). To date, only a few target genes have been identified for many of these transcription factors.

In CRT-deficient cells, inositol 1,4,5-trisphosphate (InsP\textsubscript{3})-dependent Ca\textsuperscript{2+} release from the ER is inhibited (Nakamura et al., 2001b), indicating that Ca\textsuperscript{2+}-dependent signaling pathways might be affected in the absence of CRT. Remarkably, overex-
pression of constitutively active calcineurin (activated CaN), a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, reverses the defect in cardiac development observed in CRT-deficient mice and rescues them from embryonic lethality (Guo et al., 2002). The molecular mechanisms responsible for this rescue are not known and it remains to be determined how CRT, a protein resident in the ER, impacts cardiac development.

In this study, we sought to identify the molecular mechanisms responsible for both the CRT-dependent embryonic lethality and its CaN-dependent rescue. We show that nuclear translocation of MEF2C requires CRT-dependent activation of CaN and this interplay between CRT and MEF2C is a major factor in CRT-deficient embryonic lethality. We also show that MEF2C is a potent activator of the CRT gene in a positive feedback mechanism that ensures that an adequate supply of releasable Ca\(^{2+}\) is maintained within the cell for activation of CaN and, consequently, for nuclear translocation of MEF2C. This study identifies steps in a set of apparently critical interactions among CRT, CaN, and MEF2C that occur in the early stages of cardiac development.

**Results**

**MEF2C localization and transcriptional activity are compromised in the absence of CRT**

To identify the molecular changes associated with CRT deficiency, we performed protein/DNA array analysis, which was designed for the analysis of Ca\(^{2+}\)-dependent transcription factors. To distinguish between transcription factors that are present or absent in the nucleus of wild-type and \(\text{crt}^{-/-}\) cells, in both the absence and presence of Ca\(^{2+}\), nuclear fractions were isolated from wild-type and \(\text{crt}^{-/-}\) cardiomyocytes, which had been treated with a Ca\(^{2+}\) ionophore. Initially, we tested for nuclear localization of NF-AT. NF-AT was localized to the nucleus in bradykinin-stimulated wild-type cells but was absent in the \(\text{crt}^{-/-}\) cells (Mesaël et al., 1999; Fig. 1 A). When Ca\(^{2+}\) was added to the \(\text{crt}^{-/-}\) cells, NF-AT was translocated to the nucleus (Fig. 1 A) because of a Ca\(^{2+}\)-dependent activation of CaN. Most importantly, nuclear extracts isolated from \(\text{crt}^{-/-}\) cells did not contain MEF2C (Fig. 1 A). After treatment of these cells with the Ca\(^{2+}\) ionophore the nuclear extracts did contain MEF2C (Fig. 1 A). In contrast, the nuclear localization of GATA was unaffected by the presence or absence of CRT or Ca\(^{2+}\) (Fig. 1 A).

Next, we performed a histological analysis to compare the localization of MEF2C in wild-type and \(\text{crt}^{-/-}\) embryonic day (E) 12.5 embryonic hearts. In sections from the ventricles of wild-type hearts, MEF2C was localized to the nucleus of the cardiomyocytes (Fig. 1 B, a). In contrast, in sections from \(\text{crt}^{-/-}\) embryos, MEF2C was localized predominantly in the cytoplasm (Fig. 1 B, b). To determine whether or not MEF2C is functionally active in the absence of CRT, mRNA was isolated from wild-type and \(\text{crt}^{-/-}\) cardiac tissue and then analyzed by RT-PCR to assess the expression of MEF2C gene targets (Morin et al., 2000). MLC2v, eHAND, and Irx4 mRNAs were readily detected in cardiomyocytes from wild-type animals, but not in those from \(\text{crt}^{-/-}\) animals (Fig. 1 C). Thus, we conclude that the localization of MEF2C to the nucleus and its function are both altered in the absence of CRT.

**Ca\(^{2+}\) and CaN restore the nuclear localization of MEF2C in \(\text{crt}^{-/-}\) cells**

We investigated the roles of Ca\(^{2+}\), CaN, and Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK) in the nuclear targeting of MEF2C. In wild-type cells stimulated with bradykinin (Nakamura et al., 2001b), MEF2C was localized to the nucleus (Fig. 2 A, a), in keeping with our previous observations in ventricle wall sections (Fig. 1 B, a). This localization was unaffected by Ca\(^{2+}\) or by the expression of activated CaN, activated CaMKII (Fig. 2 A, b–d), or activated CaMKI (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200412156/DC1). The nuclear import of MEF2C in \(\text{crt}^{-/-}\) cells stimulated with bradykinin was also impaired and the majority of MEF2C was found in the cytoplasm (Fig. 2 A, e). Expression of recombinant CRT in \(\text{crt}^{-/-}\)
cells restored nuclear translocation of MEF2C (Fig. 2 A, i). MEF2C was also found in the nucleus of crt⁻/⁻ cells after their treatment with a Ca²⁺ ionophore (Fig. 2 A, f). These observations indicate that the translocation of MEF2C into the nucleus depends on increased concentrations of Ca²⁺ in the cytoplasm.

We looked at possible roles for CaN and CaMKII in the Ca²⁺-dependent nuclear translocation of MEF2C. When we transfected crt⁻/⁻ fibroblasts with an expression vector encoding activated CaN, the nuclear translocation of MEF2C was fully restored (Fig. 2 A, g). The expression of activated CaMKII (Fig. 2 A, h) or activated CaMKII (Fig. S1) did not increase translocation of MEF2C to the nucleus in crt⁻/⁻ cells. The activated CaMKII expressed in these cells was functional, even in the absence of CRT, as indicated by its ability to promote the nuclear export of GFP-histone deacetylase (HDAC) 5 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200412156/DC1; McKinsey et al., 2001).

Translocation of MEF2C to the nucleus was also investigated in wild-type and crt⁻/⁻ fibroblasts transfected with expression vector encoding a GFP-MEF2C fusion protein. GFP-MEF2C was localized in the nucleus of wild-type cells and this was unaffected by Ca²⁺, activated CaN, or activated CaMKII (Fig. 2 B, a–d). In agreement with the immunostaining analysis presented in Fig. 2 A, we found that GFP-MEF2C was mainly located in the cytoplasm in crt⁻/⁻ cells (Fig. 2 B, e). We noted that GFP-MEF2C exhibited some nuclear localization in crt⁻/⁻ fibroblasts and cardiomyocytes (Fig. 2, B, D, and E). Quantitative analysis of MEF2C cellular distribution revealed that it was only detected in the nuclear fractions isolated from wild-type cells and this localization was not affected by the presence of activated CaN (Fig. 2 C). In crt⁻/⁻ cells the majority of GFP-MEF2C was recovered in the cytoplasmic fraction with a small quantity in the nuclear fraction (Fig. 2 C). The level of nuclear GFP-MEF2C in crt⁻/⁻ cells varied from 15 to 38% (Fig. 2 C) of the total GFP-MEF2C. Importantly, in crt⁻/⁻ cells expressing activated CaN, GFP-MEF2C was translocated to the nucleus and recovered only in the nuclear fraction (Fig. 2 C). It was not clear why some GFP-MEF2C was found in the nucleus.

Figure 2. Nuclear localization of MEF2C is impaired in the absence of CRT. (A) Localization of MEF2C in bradykinin-stimulated wild-type cells (a), crt⁻/⁻ cells (e), and crt⁻/⁻ cells expressing recombinant CRT (crt⁻/⁻+CRT, i). (b, f, and j) Cells were treated with Ca²⁺ ionophore. (c, g, and k) Wild-type, crt⁻/⁻, and crt⁻/⁻+CRT cells transfected with expression vector encoding activated CaN. (d, h, and l) Cells transfected with activated CaMKII expression vector. (B) Localization of GFP-MEF2C in bradykinin-stimulated wild-type cells (a–d), crt⁻/⁻ cells (e–h), and crt⁻/⁻ cells stably transfected with CRT (i–l). (b, f, and j) Cells were treated with Ca²⁺ ionophore. (c, g, and k) Wild-type, crt⁻/⁻, and crt⁻/⁻+CRT cells transfected with expression vector encoding activated CaN. (d, h, and l) Cells transfected with activated CaMKII expression vector. (C) Wild-type and crt⁻/⁻ mouse embryonic fibroblasts were transiently transfected with expression plasmids for GFP-MEF2C and activated CaN followed by Western blot analysis. wt, wild-type cells. (D) Cardiomyocytes isolated from wild-type (a and c) or crt⁻/⁻ (b and d) E12.5 embryos were cultured and transfected with expression plasmids for GFP-MEF2C and activated CaN. GFP-MEF2C was exclusively nuclear in wild-type cells stimulated with bradykinin (a) or crt⁻/⁻ cells expressing activated CaN (d), but not in crt⁻/⁻ cells (b). (E) Wild-type mouse embryonic fibroblasts were transfected with expression plasmids for GFP-MEF2C, activated CaN (CaN*), activated CaMKI (CaMKI*), and activated CaMKII (CaMKII*). Cells were treated with 0.1 μM CsA (b–d) or 1 μM KN62 (f–h).
in \(\text{crt}^{-/-}\) cells but a limited mistargeting of GFP-MEF2C has also been reported for skeletal muscle cells (Wu et al., 2000). The expression of activated CaMKII (Fig. 2 B, h) had no effect on the localization of GFP-MEF2C in \(\text{crt}^{-/-}\) cells; MEF2C remained predominantly cytoplasmic. In \(\text{crt}^{-/-}\) cells expressing recombinant CRT, the GFP-MEF2C was fully recovered in the nucleus (Fig. 2 B, i) and it remained nuclear in the presence of \(\text{Ca}^{2+}\) ions and in cells expressing activated CaN (Fig. 2 B, j and k). Inefficient nuclear targeting of GFP-MEF2C was also seen in cultured embryonic cardiomyocytes isolated from E12.5 wild-type embryos (Fig. 2 D, a and b) and some nuclear GFP-MEF2C was also detected (Fig. 2 B, e and h). Importantly, the expression of activated CaN in \(\text{crt}^{-/-}\) embryonic cardiomyocytes restored the nuclear translocation of GFP-MEF2C (Fig. 2 D, d).

To further investigate the role of CaN in MEF2C nuclear targeting, we examined MEF2C localization in wild-type cells treated with cyclosporin A (CsA), a drug that blocks CaN activity. In the presence of CsA, GFP-MEF2C was predominantly cytoplasmic and only a small fraction of GFP-MEF2C was nuclear (Fig. 2 E). This was similar to GFP-MEF2C localization in \(\text{crt}^{-/-}\) cells (Fig. 2 B) and it was in sharp contrast to exclusively nuclear localization of GFP-MEF2C in wild-type untreated cells. Expression of activated CaN, activated CaMKI, or activated CaMKII had no effect on the GFP-MEF2C localization in wild-type cells treated with CsA and the GFP-MEF2C remained largely cytoplasmic (Fig. 2 E, b–d). We also used a CaMK inhibitor, KN62, to further test if CaMK may play any role. Inhibition of CaMK had no effect on nuclear localization of GFP-MEF2C and it remained nuclear in the presence of KN62 (Fig. 2 E, e–h). Together our DNA array and immunolocalization data indicate that, in both fibroblasts and cardiomyocytes, translocation of MEF2C to the nucleus is compromised in the absence of CRT but is fully restored by increased cytoplasmic \(\text{Ca}^{2+}\) concentration or by the activation of CaN. These findings provide biochemical evidence that CRT and CaN regulate cellular targeting and activity of MEF2C.

To investigate the specificity of the CRT/CaN-dependent pathway for translocation of MEF2C, we examined whether the nuclear localization of other muscle transcription factors (GFP-MEF2A, GFP-MyoD, and GFP-GATA6) is affected by the absence of CRT. GFP-MEF2A was localized to the nucleus in both wild-type and \(\text{crt}^{-/-}\) fibroblasts (Fig. 3 A), and this was unaffected by stimulation of cells with bradykinin (not depicted), changes in the intracellular \(\text{Ca}^{2+}\) concentration with ionophore (Fig. 3 A, b), and expression of activated CaN (Fig. 3 A, c and g) or activated CaMKII (Fig. 3 A, d and h). GFP-MyoD (Fig. 3 B, a and b) and GFP-GATA6 (Fig. 3 B, c and d) were also localized to the nucleus in the \(\text{crt}^{-/-}\) fibroblasts. We also found that GFP-HDAC5 was localized to the nucleus in both wild-type and \(\text{crt}^{-/-}\) fibroblasts (Fig. S2). These data indicate that the compromised nuclear localization of MEF2C seen in \(\text{crt}^{-/-}\) cells is specific for this transcription factor.

**A role of the COOH-terminal domain of MEF2C**

We investigated whether the COOH-terminal region of MEF2C, which contains a NLS and a nuclear retention signal (NRS; Borghi et al., 2001), is responsible for \(\text{Ca}^{2+}\) - and CaN-dependent nuclear targeting of MEF2C. The GFP fusion protein that encompasses both the NRS and NLS (GFP-MEF2C-NRS-NLS; Fig. 4 A) localized to the nucleus in bradykinin-stimulated wild-type cells, and it remained there in the presence of \(\text{Ca}^{2+}\), activated CaN, or activated CaMKII (Fig. 4 B, a–d). In contrast, it was mainly cytoplasmic in the stimulated \(\text{crt}^{-/-}\) cells (Fig. 4 B, e) with a portion of the GFP fusion protein in the nucleus, just as we have seen for full-length GFP-MEF2C (Fig. 2 B). The addition of a \(\text{Ca}^{2+}\) ionophore or the expression of activated CaN both resulted in translocation of the GFP-MEF2C-NRS-NLS to the nucleus (Fig. 4 B, b–d). However, GFP-MEF2C-NLS failed to completely translocate to the nucleus in \(\text{crt}^{-/-}\) cells (Fig. 4 C, e), and its full translocation was restored by...
Ca\(^{2+}\) (Fig. 4 C, f) and by activated CaN (Fig. 4 C, g). Thus, the COOH-terminal 30 amino acids of MEF2C are essential and sufficient for its translocation to the nucleus, and they mediate the Ca\(^{2+}\)- and CaN-dependent restoration of translocation that is seen in the absence of CRT.

**MEF2C is a target of CaN phosphatase activity**

The NLS and NRS, which are located at the COOH-terminal of MEF2C, are flanked by potential phosphorylation sites (Borghi et al., 2001). To determine whether CaN affects the phosphorylation state of MEF2C, wild-type and \(crt^{-/-}\)-expressing cells GFP-MEF2C-NLS were stimulated with bradykinin followed by treatment with CsA or treatment with a Ca\(^{2+}\) ionophore. Immunoblot analysis revealed expression of two distinct protein bands: a lower mobility corresponding to hyperphosphorylated GFP-MEF2C-NLS and a greater mobility representing dephosphorylated GFP-MEF2C-NLS (Fig. 4, D and E). The identity of the hyperphosphorylated GFP-MEF2C-NLS was confirmed by incubation of protein extracts with alkaline phosphatase (Fig. 4, D and E). In the wild-type cells, the dephosphorylated form of GFP-MEF2C-NLS was detected in cells stimulated with bradykinin (Fig. 4 D, lane 3), or expressing activated CaN (Fig. 4 D). Hyperphosphorylated GFP-MEF2C-NLS was only observed in wild-type cells stimulated with CsA (Fig. 4 D, lane 2). In the absence of CRT, the dephosphorylated form of GFP-MEF2C-NLS was detected only in the presence of the Ca\(^{2+}\) ionophore (Fig. 4 E, lane 3) or in conjunction with the expression of activated CaN (Fig. 4 E, lane 4). The requirement for active CaN had no effect on the electrophoretic mobility of GFP alone (unpublished data). We conclude that the COOH-terminal region of MEF2C, containing the NLS consensus sequence important for nuclear translocation of the protein, is a target for Ca\(^{2+}\)-dependent dephosphorylation by CaN.

To identify a specific amino acid residue at the COOH terminus of MEF2C that might play a role in CaN-dependent nuclear translocation of MEF2C, we performed site-specific mutation of threonine and serine residues located near the MEF2C NLS. T409A, S412A, S418A, S420A, and T432A MEF2C mutants were expressed in \(crt^{-/-}\) cells followed by luciferase reporter gene analysis (Fig. 4 F). In agreement with the localization studies, wild-type MEF2C was fully active in bradykinin-stimulated wild-type cells but not in stimulated \(crt^{-/-}\) cells (Fig. 4 F). T409A, S418A, S420A, and T432A mutants remain inactive in the absence of CRT (Fig. 4 F). In contrast, mutation of Ser\(^{12}\) to Ala produced transcriptionally active MEF2C in \(crt^{-/-}\) cells (Fig. 4 F), indicating that Ser\(^{12}\) played a role in Ca\(^{2+}\)- and CaN-dependent activation and nuclear translocation of MEF2C.

**The CRT gene is a target for MEF2C**

Next, we investigated the possibility that MEF2C might activate the CRT gene during cardiogenesis. We cotransfected NIH3T3 cells with an expression vector encoding MEF2C and a vector containing the luciferase reporter gene controlled by MEF2C or MEF2C mutants. Cells were stimulated with bradykinin, harvested, and assayed for luciferase and β-galactosidase activity (luciferase/β-galactosidase = 891090 ± 5 and 1358402 ± 4% in wild-type and \(crt^{-/-}\) cells, respectively). The data shown are the mean ± SD of three independent experiments.

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**Figure 4.** Ca\(^{2+}\)- and CaN-dependent dephosphorylation of MEF2C. (A) Schematic diagrams of full-length MEF2C and GFP-MEF2C deletion mutants. Red box, MEF2C domain; dark blue box, MADS box. The locations of the NLS and NRS are indicated. (B) Localization of GFP-MEF2C-NLS fusion protein in mouse embryonic fibroblasts. GFP-MEF2C-NRS-NLS was nuclear in bradykinin-stimulated wild-type cells (a) and cytoplasmic in stimulated \(crt^{-/-}\) cells (d). GFP-MEF2C-NLS was translocated to the nucleus in \(crt^{-/-}\) cells, which had been treated with a Ca\(^{2+}\) ionophore (e), and in cells expressing activated CaN (f). (C) GFP-MEF2C-NLS was localized to the nuclei of stimulated wild-type cells (α–c), whereas it was present predominantly in the cytoplasm of stimulated \(crt^{-/-}\) cells (d). Increased cytoplasmic Ca\(^{2+}\) concentrations (e) and the expression of activated CaN (f) both resulted in translocation of GFP-MEF2C-NLS to the nucleus. Activated CaMII had no effect and the fusion protein remained cytoplasmic in \(crt^{-/-}\) cells. (D) Wild-type mouse embryonic fibroblasts were transfected with an expression vector for GFP-MEF2C-NLS and stimulated with bradykinin (lanes 1–3 and 5). Top and bottom protein bands correspond to hyperphosphorylated (hyperGFP-MEF2C-NLS) and hypophosphorylated (hypoGFP-MEF2C-NLS) GFP-MEF2C-NLS, respectively. (E) \(crt^{-/-}\) cells were transfected with an expression vector for GFP-MEF2C-NLS and activated CaN followed by Western blot analysis as described in D. (D and E) Lane 1, control; lane 2, cells treated with 0.25 μM CsA; lane 3, cells treated with Ca\(^{2+}\) ionophore; lane 4, cells expressing activated CaN; lanes 5–8, protein extracts treated with AP (CIP). (F) Wild-type and \(crt^{-/-}\) cells were transiently cotransfected with the expression vectors encoding MEF2C or MEF2C mutants. Cells were stimulated with bradykinin, harvested, and assayed for luciferase and β-galactosidase activity (luciferase/β-galactosidase = 891090 ± 5 and 1358402 ± 4% in wild-type and \(crt^{-/-}\) cells, respectively). The data shown are the mean ± SD of three independent experiments.
the CRT promoter. There was an induction of luciferase activity in bradykinin-stimulated NIH3T3 cells transiently transfected with an expression vector encoding MEF2C (Fig. 5 A). Although Nkx2.5, myocardin, and GATA6 also activated the CRT promoter, MEF2C had the strongest effect (Fig. 5 A). NF-ATc4 had no effect on the CRT promoter (Fig. 5 A). Cotransfection of NIH3T3 cells with expression vectors encoding MEF2C and other factors did not further increase transcriptional activation of the CRT promoter (Fig. 5 A). This finding suggests that MEF2C does not interact with other transcription factors when it activates the CRT promoter.

Deletion analysis indicated that activation of the CRT promoter by MEF2C occurs via a site located at −846 to −837 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200412156/DC1). To determine whether MEF2C bound to this element, we performed an electrophoretic mobility shift assay (EMSA). In the EMSA, we used recombinant MEF2C and ϕ32P-labeled synthetic deoxyoligonucleotides, which had either a sequence matching the putative MEF2C binding region on the CRT promoter (Fig. 5 B, probe 1) or a modified sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect MEF2C consensus sequence (Fig. 5 B, probe 2). In the probe 2, the probe 1 sequence was modified by replacing AA nucleotides with CT nucleotides to generate, potentially, a perfect
were transfected with an expression vector encoding His-tagged MEF2C, and MEF2C-DNA complexes were then immunoprecipitated with anti-His antibodies. Fig. 5 D (lane 3) shows that the immunoprecipitate contained a DNA fragment corresponding to the MEF2C binding site in the CRT promoter. Site-specific mutation of the nucleotide sequences corresponding to this binding site inhibited MEF2C-dependent activation of the CRT promoter (Fig. 5 E). Together these data indicate that MEF2C binds to the CRT promoter in living cells, fibroblasts, and cardiomyocytes and identifies a MEF2C binding site in the CRT promoter at -845 to -836. To further demonstrate that MEF2C activates the CRT gene, we investigated expression of CRT in transgenic mice overexpressing MEF2C in the heart. The MEF2C transgenic line used in this study expressed ~50% more cardiac MEF2C compared with endogenous levels (Fig. 5 F; unpublished data). Western blot analysis revealed similar increase in the level of CRT in MEF2C overexpresser transgenic hearts (Fig. 5 F). Together, our in vitro and in vivo data establish that the CRT gene is a target for MEF2C.

**CRT is necessary for maintenance of MEF2C function**

We used a luciferase reporter assay, this time to determine whether MEF2C function is compromised in the absence of CRT. Wild-type (Fig. 5 G) and crt<sup>-/-</sup> cells (Fig. 5 H) were transiently transfected with an expression vector for MEF2C and with a vector containing the luciferase gene controlled by the CRT promoter (Fig. 5, G and H) or the luciferase gene controlled by 6× MEF2C sites (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200412156/DC1). In wild-type cells, MEF2C activated the CRT promoter and this activation was enhanced by the addition of Ca<sup>2+</sup> and by activated CaN (Fig. 5 G). The expression of activated CaMKII had no effect (Fig. 5 G). In contrast, in crt<sup>-/-</sup> cells, MEF2C did not activate the reporter gene (Fig. 5 H) but it was fully restored by Ca<sup>2+</sup> and by activated CaN (Fig. 5 H). Again, CaMKII had no effect on MEF2C-dependent transcriptional activity (Fig. 5, G and H). Importantly, MEF2C-dependent activation of the luciferase gene controlled by 6× MEF2C sites was sensitive to the presence of CsA in wild-type cells, indicating involvement of CaN in MEF2C function (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200412156/DC1). These data indicate that Ca<sup>2+</sup>-dependent CaN phosphatase activity is essential in the Ca<sup>2+</sup>-dependent regulation of MEF2C activity.

**CRT enhances MEF2C activity**

Next, we asked if up-regulation of CRT expression affects MEF2C function. To do this we used the HEK293 Tet-On CRT-inducible cell line (Arnaudeau et al., 2002). Cells were transiently transfected with vector encoding luciferase reporter gene under control of 6× MEF2C binding sites. The cells were treated with doxycycline to induce expression of CRT followed by luciferase reporter assay. CRT expression was doubled after 24 h of doxycycline treatment of HEK293 Tet-On CRT cells (Fig. 6; Arnaudeau et al., 2002). Reporter gene analysis of Bradykinin-stimulated cells revealed that HEK293 Tet-On CRT cells overexpressing CRT had increased activity of MEF2C (Fig. 6). This increased activity of MEF2C was inhibited by CsA (unpublished data), supporting our conclusion that CRT affected transcriptional activity of MEF2C via the CaN-dependent pathway. Together, these findings indicate that the CRT gene is an important target for MEF2C and that increased expression of CRT enhances MEF2C transcriptional activity.

**Discussion**

In this study, we have shown that both translocation of MEF2C into the nucleus and transcriptional activation by MEF2C are compromised in the absence of CRT. These conclusions are supported by data from DNA arrays; from immunocytochemical localization of MEF2C, and GFP-MEF2C, in CRT-deficient fibroblasts, in crt<sup>-/-</sup> cardiomyocytes in culture, and in the embryonic heart; and from RT-PCR showing reduced expression of known MEF2C target genes in embryonic hearts. The translocation of MEF2C and its activation of transcription are restored in CRT-deficient cells by the expression of CRT, Ca<sup>2+</sup>-dependent activation of CaN, and activated CaN. These effects of CRT on MEF2C are specific to this protein because nuclear targeting of the transcription factors MEF2A and MEF2D, MyoD, and GATA6 is unaffected in the absence of CRT, indicating that the nuclear translocation machinery is still functional.

The transcription factor MEF2C plays an important role in the control of cardiac and skeletal muscle development (McKinsey et al., 2002; Frey and Olson, 2003). MEF2C is the first of the MEF2 family of genes to be expressed during cardiac development, with transcripts appearing in mesodermal...
phorylation of transcriptional repressors (HDACs; McKinsey has been reported to occur indirectly, mainly through the phosphorylation of activated CaMKI or activated CaMKII. Some synergism among MEF2C, activated CaN, and activated CaMKIV has previously been reported in skeletal muscle (Wu et al., 2000), but in this study activated CaMKI and activated CaMKIV had no effect. CaMK-dependent activation of MEF2C has previously been reported to occur indirectly, mainly through the phosphorylation of transcriptional repressors (HDACs; McKinsey et al., 2000).

CaN is a highly conserved, Ca\(^{2+}\)/calmodulin-dependent serine/threonine phosphatase (Aramburu et al., 2000), and its role in the NF-AT signaling pathway in many tissues is well established (Crabtree, 2001). This pathway plays a role in normal cardiac physiology and in cardiac pathologies (Molkentin et al., 1998; Wilkins and Molkentin, 2002). CaMK-dependent activation of MEF2C has been reported to occur indirectly, mainly through the phosphorylation of transcriptional repressors (HDACs; McKinsey et al., 2000).

Figure 7. A model of the Ca\(^{2+}\)-dependent signaling cascade from the ER to the nucleus. The model shows cross talk between CRT, Ca\(^{2+}\), CaN, and MEF2C. CRT is a Ca\(^{2+}\)-buffering ER protein that affects Ca\(^{2+}\) storage in the ER, Ca\(^{2+}\) release from the ER, and store-operated Ca\(^{2+}\) influx. Ca\(^{2+}\) is released from the ER resulting in an increased intracellular Ca\(^{2+}\) concentration to activate CaN. This results in dephosphorylation of the COOH-terminal region of MEF2C, promoting its translocation to the nucleus.

Several precursor cells that give rise to the heart (Lin et al., 1997; Black and Olson, 1998). MEF2C plays a key role in the activation of several fetal cardiac genes (Morin et al., 2000), and we found that in the absence of CRT there is no significant expression of MLC2v, eHAND, and Irx4, indicating that transcriptional activation by MEF2C requires CRT. DNA arrays and immunolocalization studies have shown that localization to the nucleus of both NF-AT and MEF2C is impaired in the absence of CRT. Because localization of MEF2C to the nucleus was restored by increased cytoplasmic Ca\(^{2+}\) concentrations, we sought to identify which Ca\(^{2+}\)-dependent cytoplasmic enzymes could influence MEF2C localization and/or activity. Studies monitoring nuclear localization and expression of the luciferase reporter gene suggest that MEF2C function is enhanced by coexpression of activated CaN, but that it is not influenced by coexpression of activated CaMKI or activated CaMKII. Some synergism among MEF2C, activated CaN, and activated CaMKIV has previously been reported in skeletal muscle (Wu et al., 2000), but in this study activated CaMKI and activated CaMKIV had no effect. CaMK-dependent activation of MEF2C has been reported to occur indirectly, mainly through the phosphorylation of transcriptional repressors (HDACs; McKinsey et al., 2000).

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cell and organ functions, including cardiogenesis (Webb and Miller, 2003). To activate Ca\(^{2+}\)-dependent signaling pathways, Ca\(^{2+}\) is released from the ER and comes from the extracellular space leading to increase in cytoplasmic Ca\(^{2+}\) concentration affecting multiple Ca\(^{2+}\)-dependent processes (Berridge et al., 2003). During cardiac development an important target protein is CaN (Fig. 7), which, when activated by a sustained increase in intracellular Ca\(^{2+}\) concentration, dephosphorylates MEF2C, promoting its translocation from the cytoplasm into the nucleus (Fig. 7). Among its many effects, Ca\(^{2+}\) release from the ER also activates CaMKs. These kinases are known to influence MEF2C function in the heart via phosphorylation of a specific sequence, via CaN (Fig. 7). Importantly, this positive feedback pathway, between NF-AT, TRPC3, and CaN in skeletal muscle. In conclusion, one of the most interesting findings in the present study is that the CRT gene is itself a target for transcriptional activation by MEF2C in developing cardiac muscle. Using ChIP analysis, we found that MEF2C binds to the CRT promoter in vivo. Most importantly, up-regulation of CRT expression has a significant positive effect on MEF2C transcriptional activity, illustrating a positive feedback mechanism whereby MEF2C controls the expression of an ER Ca\(^{2+}\)-binding protein to ensure a sufficient supply of "releasable" Ca\(^{2+}\) for its own activation, via CaN (Fig. 7). Importantly, this positive feedback pathway also ensures that the Ca\(^{2+}\)-dependent activity of MEF2C is preserved. Rosenberg et al. (2004) reported that function of NF-AT is regulated by neuromuscular stimulation in skeletal muscle and that Ca\(^{2+}\) entry via TRPC3 channel is critical for this activity. Interestingly, expression of TRPC3 in skeletal muscle is up-regulated by the CaN/NFAT-dependent pathway (Rosenberg et al., 2004). A positive feedback mechanism between MEF2C, CRT, and CaN in the heart may also exist between NF-AT, TRPC3, and CaN in skeletal muscle. In conclusion, the present findings identify important functional relationships between CRT, CaN, and MEF2C in the early stages of cardiac development in vivo, and they indicate that the failure of these interactions is a major cause of the embryonic lethality in CRT-deficient mice. Other CaN-dependent pathways including activation of NF-AT may also contribute to the embryonic lethality observed in the absence of CRT (Mesa1 et al., 1999).

In vertebrates, expression of MEF2C in the heart occurs very early on in development, at E7.5 (Lints et al., 1993; Edmondson et al., 1994; Molkentin et al., 1996). At E9.5 the expression of MEF2C is observed throughout the cardiac atria and ventricles, and it continues to be highly expressed until E14.5. This pattern of expression is highly similar to that observed for CRT in the developing myocardium (Mesa1 et al., 1999). Further, after birth there is a parallel decline in the cardiac expression of both MEF2C and CRT, and, conversely, during cardiac hypertrophy in adults both genes are up-regulated (Edmondson et al., 1994). This last observation indicates that MEF2C-dependent activation of the CRT gene, and CRT/CaN-dependent functional regulation of MEF2C, may also play an important role in adult cardiac pathology.

The CRT gene is a target for the transcription factors Nkx2.5 (Guo et al., 2001) and myocardin, which are expressed as early as E7.5 in the embryonic heart. Therefore, the sequence of events during cardiac development seems to be a very early activation of the CRT gene, by Nkx2.5 and other cardiac-specific transcription factors, followed by the expression of MEF2C at E9.5 and the subsequent activation of several cardiac-specific genes, including the CRT gene. The CRT gene is an extremely important target for MEF2C because, at these stages in cardiac development, CRT plays a critical role in enabling MEF2C to function. In summary, the data presented here identify an important role for CRT and CaN in transduction of the Ca\(^{2+}\) signaling pathways, which activate the transcription factor MEF2C in the developing myocardium. They also highlight a previously unrecognized positive feedback loop, whereby CRT modulates MEF2C activity and MEF2C regulates the expression of CRT.

Materials and methods

Plasmid DNA

Expression vectors containing cDNA encoding MEF2C, GFP-HDAC5, activated CaMK, and activated CaMkI were obtained from E. N. Olson (University of Texas Southwestern Medical Center, Dallas, TX); MEF2A and MEF2D cDNAs from J. McDermott (York University, Toronto, Canada); Nkx2.5 from I. Komuro (University of Tokyo, Tokyo, Japan); GATA6 from M. Nemer (Institut de Recherches Cliniques de Montreal, Montreal, Canada); NFATc4 from T. Hoey (Tularik, San Francisco, CA); MyoD from M. Puceat (Centre National de la Recherche Scientifique, Montpellier, France); and activated CaMKII from H. Schuman (Stanford University, Stanford, CA).

To generate a MEF2C expression vector (pcDNA-MEF2C-myc-His), a cDNA fragment encoding 432 aa residues of mouse MEF2C was amplified by a PCR-driven reaction and cloned into the XhoI and BamHI sites of pcDNA3.1/myc-His. cDNAs encoding full-length or parts of proteins were generated by PCR-driven reaction and subcloned into the pEGFP-C1 expression vector to generate GFP fusion proteins; construction of GFP-NTFt4 has been described previously (Guo et al., 2002). Full-length MEF2C cDNA was cloned into the Xhol and HindIII sites of pEGFP-C1 plasmid to generate GFP-MEF2C expression vector; the full-length MEF2A cDNA was cloned into the Xhol and EcoRI sites of pEGFP-C1 to create GFP-MEF2A; the MyoD cDNA was cloned into EcoRI and Xhol sites of pEGFP-C1 to generate GFP-MyoD; the MEF2C-NRS-NLS cDNA encoding the COOH-terminal region was cloned into the Xhol and BamHI sites of pEGFP-C1; a MEF2C-NLS encoding DNA was made synthetically and cloned into the Xhol and sites of pEGFP-C1 plasmid; the NLS region of GATA6 was isolated by SfiI and EcoRI digestion of GATA6 cDNA and then cloned into the pEGFP-C1 vector to generate a pGFP-GATA6-NLS expression vector.

DNA array and RT-PCR analyses

Cells were transiently transfected with pcDNA-MEF2C-myc-His expression vector using FuGENE 6 system and treated with bradykinin to induce InsP\(_3\)-dependent Ca\(^{2+}\) release from the ER before harvesting (Nakamura et al., 2001b). cft\(^{-/-}\) fibroblasts were also treated with 1 \(\mu\)M ionomycin for 4.5 h before harvesting. The nuclear proteins were isolated and then applied to Transignal protein/DNA arrays (Panomics Inc.). RNA was isolated from E12.5 wild-type and cft\(^{-/-}\) embryos followed by cDNA synthesis (Li et al., 2002). cDNA products were analyzed by step-down PCR using the following primers: for MLC2v, 5'-CCCTGAGTTATGCTACGAGAAGGC-3' forward and 5'-GGTGCACTCATCCCGAGCATAGTC-3' reverse primers; for eHAND (GenBank/EMBL/DBJ accession no. S79216); 5'-GGGGAGCCTACCGAGCCAGC-3' forward and 5'-GCTGTCGATCTCTGGCCAGC-3' reverse primers; and
for Irx4, 5'-GGCAGCTGCAGGCGTTATG-3' forward and 5'-GGCCGTCTTGCAAGTCTCC-3' reverse primers. RT-PCR analysis was performed as a control using the forward 5'-GAGTGAGTGGTGGCAGACAG-3' and reverse 5'-GGACCACTGCTTACCC-3' primers.

Western blotting and immunohistochemistry

For quantitative analysis of nuclear and cytoplasmic distribution of GFP-MEF2C, wildtype and crT mouse embryonic fibroblasts were transiently transfected with plasmid DNA containing cDNA encoding MEF2C, treated with bradykinin (Nakamura et al., 2001a, and 2001b), and harvested. Cytoplasm and nuclear proteins were isolated 48 h after transfection using an extraction kit (Panomics Inc.). Protein extracts (30 µg) were loaded onto a 10% SDS-PAGE gel, transferred to nitrocellulose membranes, and blotted with polyclonal goat anti-GFP (1:5,000; a gift from L. Berthiaume, University of Alberta, Edmonton, Alberta, Canada) followed by a peroxidase-conjugated rabbit anti-goat secondary antibody (1:10,000). Immunoblotting analysis of cultured cells and mouse embryos was performed as described previously (Messa et al., 1999). Polyclonal rabbit anti-MEF2C (Cell Signaling) was used at 1:70 dilution followed by a donkey anti-rabbit Texas red (1:70). Densitometric analysis of MEF2C was performed (Wu et al., 2000) using crT fibroblasts transiently cotransfected with expression vectors encoding activated CaN and GFP-MEF2C-NRS-NLS or GFP-MEF2C-NLS. Western blot analysis was performed with goat anti-GFP antibodies (1:5,000). Mouse mAb to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used at 1:2,000. Both images were examined with a microscope (model Axiovert 100M; Carl Zeiss MicroImaging, Inc.), captured, and pseudocolored in Northern Eclipse 5.0 software. cDNA nucleotide sequences were confirmed by nucleotide sequence analysis performed at the DNA Core Facility at the Department of Biochemistry, University of Alberta.

Online supplemental material

For mutation and deletion analysis, a plasmid containing the CRT promoter was used. The following 3'-AAAATAATC-3' (nt -845 to -836) to 5'-CCGGAATTCC-3' forward and reverse PCR primers were used for all reactions (5'-AAAATAATC-3'). The vector was digested and a fragment containing the promoter and cDNA fusion was purified for injection into newly fertilized oocytes for the standard generation of transgenic mice. Mouse mAb to GAPDH was purchased from Ambion and was used at a concentration of 1:2,000. Secondary antibody was rabbit anti-mouse HRP at a dilution of 1:12,000. NIH/3T3 fibroblasts, wildtype embryonic fibroblasts, and CRT-deficient embryonic fibroblasts were maintained as described previously (Nakamura et al., 2001b). HEK293 and HEK2993 Tet-ON CRT cells were cultured as previously described (Arnaudeau et al., 2002). Primary cardiomyocyte cultures were obtained from day 12.5 mouse embryos as described previously (Guo et al., 2001). Protein concentration was measured as described previously (Bradford, 1976). Mouse genotyping was performed by a PCR-driven amplification of genomic DNA (Guo et al., 2002). All images were examined with a microscope (model Axiovert 100M; Carl Zeiss MicroImaging, Inc.), captured, and pseudocolored in Northern Eclipse 5.0 software. cDNA nucleotide sequences were confirmed by nucleotide sequence analysis performed at the DNA Core Facility at the Department of Biochemistry, University of Alberta.

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