Calreticulin Is Essential for Cardiac Development

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Abstract. Calreticulin is a ubiquitous Ca^{2+} binding protein, located in the endoplasmic reticulum lumen, which has been implicated in many diverse functions including: regulation of intracellular Ca^{2+} homeostasis, chaperone activity, steroid-mediated gene regulation, and cell adhesion. To understand the physiological function of calreticulin we used gene targeting to create a knockout mouse for calreticulin. Mice homozygous for the calreticulin gene disruption developed omphalocele (failure of absorption of the umbilical hernia) and showed a marked decrease in ventricular wall thickness and deep intertrabecular recesses in the ventricular walls. Transgenic mice expressing a green fluorescent protein reporter gene under the control of the calreticulin promoter were used to show that the calreticulin gene is highly activated in the cardiovascular system during the early stages of cardiac development. Calreticulin protein is also highly expressed in the developing heart, but it is only a minor component of the mature heart. Bradykinin-induced Ca^{2+} release by the $InsP_3$-dependent pathway was inhibited in $crt^{-/-}$ cells, suggesting that calreticulin plays a role in Ca^{2+} homeostasis. Calreticulin-deficient cells also exhibited impaired nuclear import of nuclear factor of activated T cell (NF-AT3) transcription factor indicating that calreticulin plays a role in cardiac development as a component of the Ca^{2+}/calcineurin/NF-AT/GATA-4 transcription pathway.

Key words: cardiac development • NF-AT • calcineurin • endoplasmic reticulum • calcium binding protein • transcription

THE ER performs an important role in controlling different intracellular processes including: protein synthesis, folding and modification, synthesis of membrane lipids, and regulation of Ca^{2+} storage and release. The ER lumen contains a characteristic set of resident proteins that are involved in many aspects of ER function, including Ca^{2+} binding and storage. One of the major Ca^{2+} binding chaperone proteins of the ER is calreticulin (Michalak et al., 1992). Calreticulin is an unusual lumenal ER protein. The protein contains both high affinity and high capacity Ca^{2+} binding sites (Ostwald and MacLennan, 1974; Baksh and Michalak, 1991) and has been implicated in the regulation of cytoplasmic Ca^{2+} homeostasis, even though it is located in the ER lumen (Liu et al., 1994; Bastianutto et al., 1995; Camacho and Lech-

leiter, 1995; Mery et al., 1996; Coppolino et al., 1997; Fasolato et al., 1998; John et al., 1998).

This regulatory function may be mediated by different Ca^{2+} binding sites in calreticulin (Baksh and Michalak, 1991; Krause and Michalak, 1997) and by the interaction of calreticulin with inositol 1,4,5-trisphosphate $(InsP_3)^1$ receptors and/or SERCA molecules (John et al., 1998). Other functions have been demonstrated for calreticulin including: modulation of gene expression (Burns et al., 1994; Dedhar et al., 1994; Michalak et al., 1996), chaperone activity (Nigam et al., 1994; Nauseef et al., 1995; Helenius et al., 1997), and regulation of cell adhesion (Coppolino et al., 1995, 1997; Opas et al., 1996). Calreticulin has also been reported to play a role in replication of Rubella virus RNA (Singh et al., 1994), in cytotoxic T-cell function/activation (Burns et al., 1992; Dupuis et al., 1993; Andrin et al., 1998), in neutrophils (Stendahl et al., 1994), in sperm

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1. *Abbreviations used in this paper:* ES cells, embryonic stem cells; GFP, green fluorescent protein; $InsP_3$, inositol 1,4,5-trisphosphate; NF-AT, nuclear factor of activated T cell.

cell function (Nakamura et al., 1993), and in autoimmunity (Sontheimer et al., 1993).

The aim of this study was to investigate the physiological functions of calreticulin by generating a calreticulin-deficient mouse. The homozygous calreticulin knockout was an embryonic lethal because of defects in heart development and function. This was a surprising result because calreticulin is an ER lumenal protein and only a minor component of the mature heart (Fliegel et al., 1989a,b; Milner et al., 1991; Tharin et al., 1996). However, we generated transgenic mice expressing the green fluorescent protein (GFP) under the control of calreticulin promoter and showed that the calreticulin gene was activated during early stages of embryonic development. Furthermore, calreticulin-deficient cells have inhibited bradykinin-induced Ca^{2+} release from the ER and impaired nuclear import of the NF-AT3 transcription factor.

Materials and Methods

Construction of the Calreticulin Knockout Vector

The plasmid pNTK (containing the PGK neomycin cassette and PGK thymidine kinase) was used to generate the knockout vector. The calreticulin promoter was cloned previously from a mouse liver genomic library (Waser et al., 1997) and digested with SalI and HindIII restriction endonucleases. Afterwards, the 1.5-kb fragment was blunt ended and ligated into the pNTK plasmid to generate the pNTC1 plasmid. The plasmid pCM101 containing the full-length mouse calreticulin gene was cut with EcoRV. Furthermore, an 11-kb fragment, containing part of exon 4 and exons 5–9 and 3-kb of 3′ flanking region, was ligated with the NotI/SalI cut and blunted pNTC1 plasmid that resulted in the calreticulin gene knockout construct pNTC6 (see Fig. 1 A). In the pNTC6 plasmid the PGK NEO cassette replaced the first four exons of the calreticulin gene, thus removing the proposed transcription initiation site, the ATG start codon, and interrupting translation of the protein.

Electroporation, Selection, and Screening of ES Cell Clones

J1 129/Sv embryonic stem (ES) cells (5×10^7) were electroporated with 100 μg of linearized pNTC6 vector using a Bio-Rad Gene Pulser at 400 V/cm and 25 μF. Cells were plated on mitomycin C–treated G-418–resistant mouse embryonic fibroblast feeder cells. Recombinant clones were selected with G418 (0.2 mg/ml) and gancyclovir (2 μM). 200 colonies were picked after 10 d in selection medium and expanded. 100 clones were screened for a homologous recombination event by PCR using the Expand Long Template PCR System (Boehringer Mannheim). The efficiency of homologous recombination was 1 in 30 clones. The heterozygote calreticulin knockout ES cells were identified by PCR using one 5′ primer (5′-GCTGGTCAAGTGTGATTCTCATGTTCCTGCCTG-3′) and two different 3′ primers (5′-CTCTGACCTTCACACTAGACACCCTTC-ATC-3′ for the wild-type gene and 5′-CTCTGACCTTCACACTAGA-CACCCTTCATC-3′ for the knockout allele) and confirmed by Southern blot analysis. These ES cells were microinjected into 3.5-d-old C57BL/6J blastocysts to generate chimeric mice. Chimeric males were analyzed for germline transmission by mating with C57BL/6J females, and the progeny were analyzed by PCR and Southern blot. For PCR amplification of the genomic DNA of knockout mice, two sets of primers were utilized (see Fig. 1 A). One resulted in detection of the wild-type gene (the primer sequences were: 5′-GAAGATCTAAACCAGTCAAAAGGACC-3′ and 5′-CTCCAGGTCCCCGTAAAATTTGCC-3′) and the second resulted in detection of the targeted knockout construct (the primer sequences were: 5′-CAGAGATCTCAGCAGCAAGGGC-3′ and 5′-CTCTGACC-TTCACACTAGACACCCTTCATC-3′). EcoRI-digested genomic DNA was used for Southern blot detection of the calreticulin gene and for detection of the targeted mutant allele using the 5′ DNA probe indicated in Fig. 1 A.

Generation of the GFP Reporter Gene Vector

The plasmid pS65T-C1 containing cDNA encoding GFP was purchased from CLONTECH Laboratories, Inc. The nucleotide sequence corresponding to the CMV promoter of this construct was removed using AseI and NheI restriction sites and the ends were blunted using Klenow polymerase. The CMV promoter was replaced with a 2.3-kb mouse calreticulin promoter (Waser et al., 1997), and cut with SmaI and StuI to generate blunt ends. The 3.58-kb vector containing the calreticulin promoter, GFP cDNA, and the SV-40 polyA site is referred to as the pCPGF construct. To generate transgenic mice, pCPGF was linearized with NaeI/SpeI (see Fig. 3 A), purified, and injected into the fertilized oocytes from the FVB/N mice (Taketo et al., 1991). Afterwards cells were transferred into pseudopregnant FVB/N mice. Genomic DNA was isolated from tail biopsies of each of the transgenic mouse litters and the presence of the GFP reporter targeting vector was detected by PCR using a 5′ primer in the CRT promoter region (5′-GATTCCTTCTGGGCAGTTCATAGTC-3′) and a 3′ primer in the GFP protein cDNA (5′-ATCTAATTCAACAA-GAATTGGGACAA-3′). The locations of these primers are indicated in Fig. 3 A. Transgenic animals and calreticulin-deficient mice were generated in the Transgenic Facility (University of Alberta Health Sciences Laboratory Animal Services, Edmonton, Alberta, Canada).

Histological Analysis

Mouse embryos, at different gestational age, were dissected out of the uterus and fixed in 4% paraformaldehyde for 30–90 min. The embryos were embedded in 30% sucrose overnight at 4°C, washed once in PBS for 1 h, placed in 50% Tissue Tek OCT compound (mounting media) in PBS saline for 8 h at room temperature, and incubated in 100% OCT overnight at 4°C. The embryos were frozen in 2-methylbutane cooled in liquid nitrogen. Cryostat sections, 8–10 μm thick, were prepared in transverse and sagittal sections. The sections were either mounted in mounting media containing 0.2% DABCO (for fluorescence analysis) or processed for immunohistochemistry and standard hematoxylin and eosin staining. A confocal microscope (MRC600; Bio-Rad Laboratories) was used to obtain images at 10× or 60× and images were reconstructed using the Adobe Photoshop program. For better visualization, simulated phase-contrast images of each fluorescent image were generated using the Adobe Photoshop program.

Immunohistochemistry

Sections from wild-type and transgenic mouse embryos were stained with appropriate antibodies followed by staining using Vectastain Elite ABC kit and Vector DAB substrate kit (Vector Labs Inc.). Primary antibodies were polyclonal rabbit antibodies to GFP (1:150 dilution; CLONTECH Laboratories, Inc.) and affinity purified anticalreticulin (1:10 dilution) (CRT283 antibody described in Michalak et al., 1996). The sections were counterstained with hematoxylin.

Activation of NF-AT3 in Mouse Embryonic Fibroblasts

Embryos from two litters of heterozygous crosses were used to derive mouse embryonic fibroblasts. Embryos $crt^{-/-}$ and wild-type genotype were dissociated, washed, trypsinized for 30 min, and cultured in 6-well tissue culture plates. Cells were maintained in DME containing 20% FCS. For transfection experiments, plasmid DNA was purified by column chromatography (Qiagen Inc.). Cells were transfected transiently with NF-AT3 expression vector alone, or cotransfected with NF-AT3 and calreticulin expression vectors as described by Burns et al. (1994). After 1 d, cells were stimulated with 200 nM bradykinin (Waser et al., 1997) followed by indirect immunofluorescence with monoclonal anti–NF-AT (mAb 7A6, a gift of G.R. Crabtree, Stanford University) and polyclonal goat anticalreticulin antibodies (Milner et al., 1991). The secondary antibodies were: Texas red–conjugated sheep anti–mouse (diluted 1:50 in PBS) and FITC-conjugated donkey anti–goat (used at 1:50 dilution). For double labeling, all incubations were carried out sequentially. A confocal fluorescence microscope (MRC-600; Bio-Rad Laboratories) equipped with a krypton/argon laser light source was used.

Ca^{2+} Measurements

Wild-type and calreticulin-deficient mouse embryonic fibroblasts (1.5×10^6 cells/ml) were loaded with fura-2/AM (Molecular Probes, Inc.). Fluo-

rescence measurements were carried out as described by Mery et al. (1996). Ca^{2+} release from internal stores was induced with either 1 μM thapsigargin (Sigma Chemical Co.), or 200 nM bradykinin (Sigma Chemical Co.). Changes in the cytoplasmic Ca^{2+} concentration were monitored in Ca^{2+}-free media (Mery et al., 1996).

Miscellaneous

Proteins were separated by SDS-PAGE on 10% polyacrylamide gels as described by Laemmli (1970), transferred to nitrocellulose membranes, and stained by immunoblotting with affinity purified rabbit anticalreticulin antibody (Michalak et al., 1996).

Genomic DNA was isolated from mouse tissue as described by Ausubel et al. (1989) and digested with EcoRI. The DNA was separated by electrophoresis on 0.8% agarose gel and transferred to Hybond-N membranes (Amersham Pharmacia Biotech). The disruption of the calreticulin gene was characterized by Southern blotting (Ausubel et al., 1989).

Results

Calreticulin Knockout

Fig. 1 A summarizes the gene targeting strategy used to generate the calreticulin gene knockout mice. During the process of homologous recombination, the PGK NEO cassette was inserted into the calreticulin gene, replacing the first four exons, thus removing the initiator ATG and interrupting the expression of calreticulin protein (Fig. 1 A). PCR analysis of genomic DNA using the specific sets of primers depicted in Fig. 1 A helped to identify the genotype of the mice. Analysis of genomic DNA by Southern blotting (Fig. 1 B) showed two hybridizing bands in genomic DNA from $crt^{+/-}$ mice corresponding to the wild-type allele (5.7 kb) and the targeted knockout allele (3.8 kb), whereas Southern blotting of genomic DNA isolated from $crt^{-/-}$ mice (Fig. 1 B) showed only one DNA band of 3.8 kb corresponding to the size of the targeted knockout gene. Western blot analysis revealed that the interruption of a single allele in $crt^{+/-}$ mice resulted in a significant decrease in calreticulin protein level, whereas in the calreticulin gene knockout animals ($crt^{-/-}$) there was no detectable expression of the protein (Fig. 1 C). $crt^{+/-}$ mice contained ~50% lower level of calreticulin protein than wild-type mice, as estimated by densitometry. Identical results were obtained with three different anticalreticulin antibodies (not shown).

Phenotype of Calreticulin Knockout Mice

Chimeric male mice were crossed with wild-type females to generate first generation heterozygotes. The $crt^{+/-}$ mice had normal phenotype, being viable and fertile. Intercrossing of the $crt^{+/-}$ males with $crt^{+/-}$ females was carried out to generate homozygote ($crt^{-/-}$) gene knockout mice. We were unable to obtain any viable $crt^{-/-}$ pups from this cross. Living $crt^{-/-}$ embryos were obtained at 18 d and earlier. In addition, a number of 12.5–16.5-d-old embryos were dead. Analysis of embryos at or after day 14.5 showed a deficit number of $crt^{-/-}$ embryos (15% instead of 25%), indicating that a significant fraction of $crt^{-/-}$ embryos died earlier. We concluded that the homozygote ($crt^{-/-}$) gene knockout was embryonic lethal and that calreticulin is essential for survival.

Accordingly, serial timed matings were set up to follow
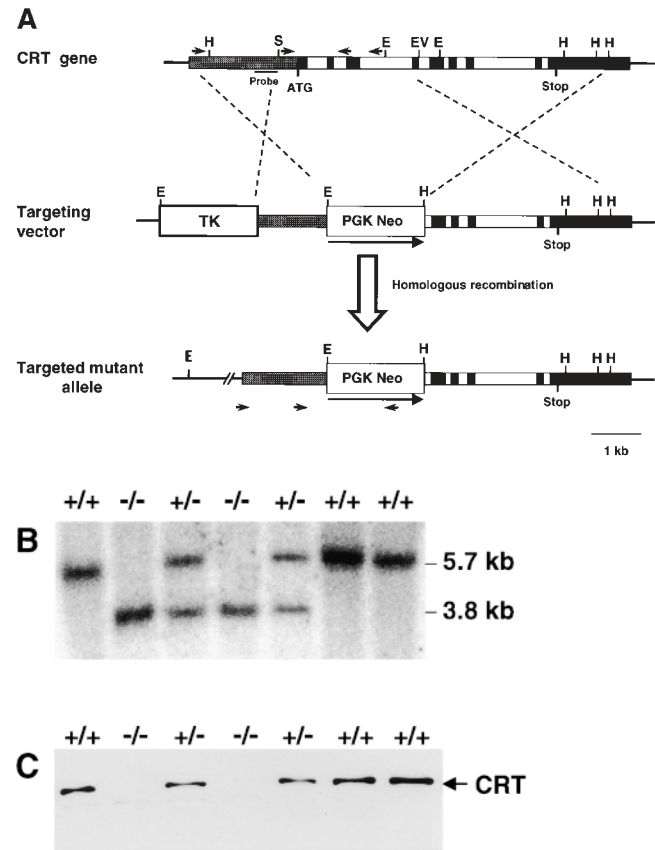
Figure 1. Targeted disruption of the calreticulin gene. (A) Top shows the structure of the mouse calreticulin gene, indicating the ATG start codon and the restriction enzymes utilized (E, EcoRI; EV, EcoRV; H, HindIII; S, SalI). The arrows indicate the PCR primer sets (see Materials and Methods) used for recognition of the wild-type gene. The solid bar is the 5' probe used for Southern blotting. Middle is the targeting vector designed to replace the first exons with the PGK NEO cassette. The targeted calreticulin allele is shown (bottom). The arrows indicate the PCR primers used for recognition of the mutant allele. (B) Southern blot of the EcoRI-digested genomic DNA isolated from wild-type (+/+), heterozygote (+/−), and homozygote (−/−) mice. The sizes of the wild-type (5.7 kb) and mutant allele (3.8 kb) are indicated. (C) Western blot analysis of proteins extracted from the biopsies of wild-type (+/+), heterozygote (+/−), and homozygote (−/−) mouse embryos. The blot was probed with the affinity-purified rabbit anticalreticulin antibody (Michalak et al., 1996).

embryonic development in an attempt to find the cause of death of the $crt^{-/-}$ mice. Fig. 2, A and B, shows photographs of 18-d-old $crt^{+/-}$ (left) and $crt^{-/-}$ (right) mouse embryos. At this level of analysis the most visible difference between the $crt^{+/-}$ and $crt^{-/-}$ embryos was the failure of absorption of the umbilical hernia (omphalocele) in the $crt^{-/-}$ embryos (Fig. 2, A and B, arrow). Histological analysis of $crt^{-/-}$ mice confirmed the omphalocele and the presence of midgut in the umbilical hernia (Fig. 2 B). The other significant defect was observed in morphology of the hearts of the $crt^{-/-}$ mice (Fig. 2 B). Fig. 2 B shows that there was a marked decrease in the thickness of the

A crt +/-   crt -/-

B crt +/-   crt -/-

C wt   crt -/-
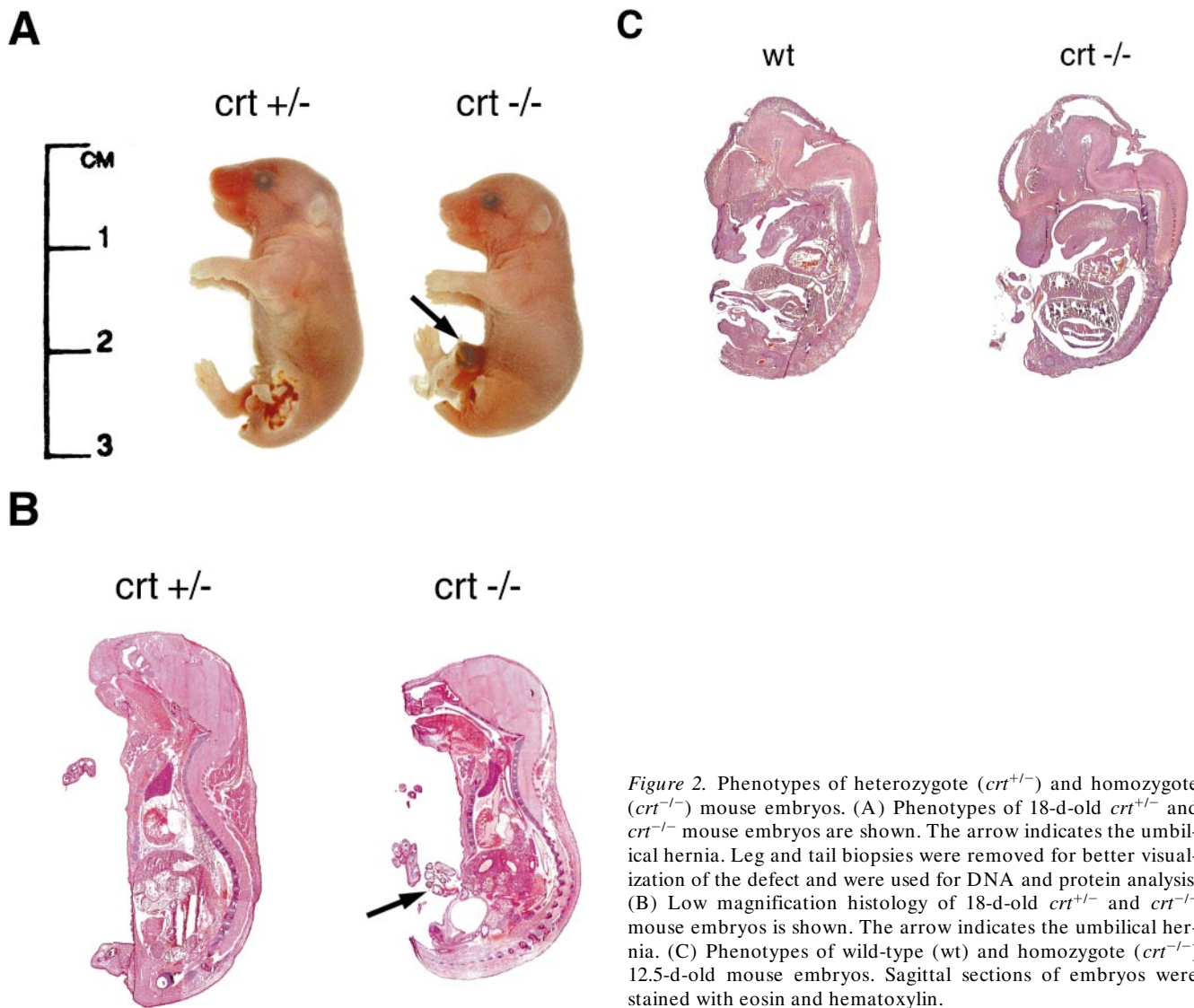
Figure 2. Phenotypes of heterozygote ($crt^{+/-}$) and homozygote ($crt^{-/-}$) mouse embryos. (A) Phenotypes of 18-d-old $crt^{+/-}$ and $crt^{-/-}$ mouse embryos are shown. The arrow indicates the umbilical hernia. Leg and tail biopsies were removed for better visualization of the defect and were used for DNA and protein analysis. (B) Low magnification histology of 18-d-old $crt^{+/-}$ and $crt^{-/-}$ mouse embryos is shown. The arrow indicates the umbilical hernia. (C) Phenotypes of wild-type (wt) and homozygote ($crt^{-/-}$) 12.5-d-old mouse embryos. Sagittal sections of embryos were stained with eosin and hematoxylin.

ventricular wall in $crt^{-/-}$ as compared to $crt^{+/-}$ mice. Histological analysis of 12.5-d-old $crt^{-/-}$ embryo also revealed defects in morphology of the heart (Fig. 2 C), indicating that calreticulin is essential for cardiac development at relatively early stages of cardiogenesis. No other gross morphological changes were detected in $crt^{-/-}$ mice (Fig. 2). Higher magnification analysis of hearts from 12.5-, 14.5-, and 18-d-old embryos revealed deep intertrabecular recesses and increased fenestration that were associated with the thinner ventricular wall (Fig. 3). However, no significant changes in the histology of the atrial wall was observed (Figs. 2 and 3). At high magnification, pictures of the ventricular wall of 18-d-old $crt^{-/-}$ embryo further demonstrate all of the following: increased fenestration, thinner ventricular wall, and the impaired growth of the compact layer of the ventricles as compared to the $crt^{+/-}$ embryos (Fig. 4).

Developmental Activation of the Calreticulin Promoter

The mature heart contains a very low level of calreticulin

(Fliegel et al., 1989a,b; Milner et al., 1991; Tharin et al., 1996) but histological analysis of calreticulin-deficient mice suggests an essential role for the protein in cardiac development. To answer the question of whether the calreticulin gene is activated during cardiac development we studied the activation of its promoter in transgenic mice expressing the GFP reporter gene under control of the calreticulin promoter. Fig. 5 A illustrates the strategy used to construct the reporter transgene. The calreticulin promoter (2.3 kb) was introduced upstream of the GFP reporter gene. Genomic DNA from the transgenic mice was analyzed by PCR for the presence of the GFP transgene (Fig. 5 B). Three separate transgenic founder mice (L9, L13, and L17) were generated and all three gave identical results. Activation of the calreticulin promoter was monitored by detection of the fluorescent signal obtained from calreticulin promoter-driven expression of the GFP reporter gene. The first generation (F1) male transgenic mice were used in serial timed matings with wild-type FVB/N females. Embryos were harvested at different gestation times, fixed, and frozen. Every embryo was tested
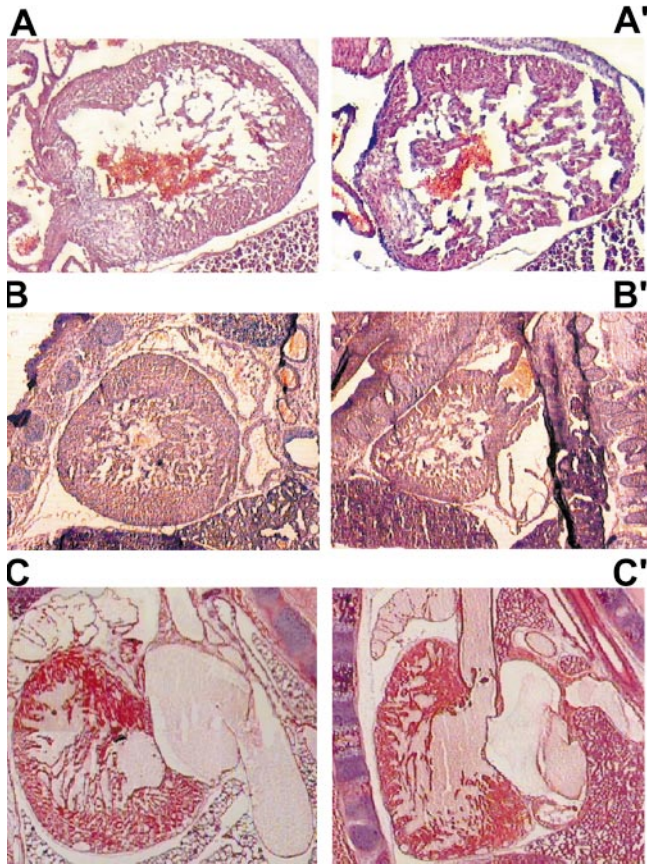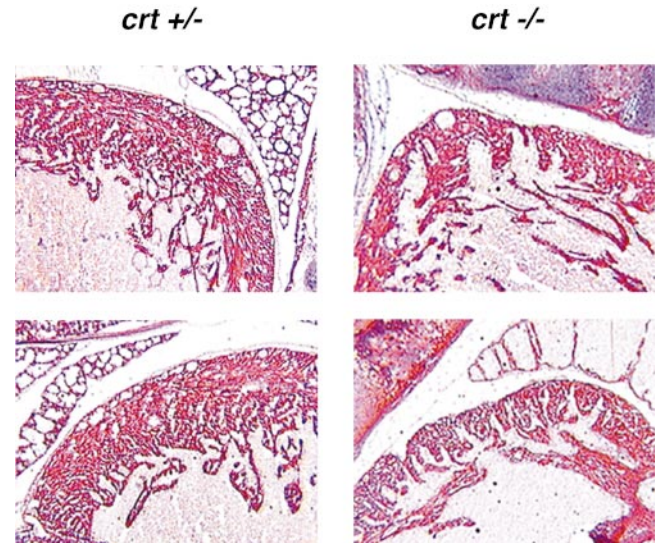
Figure 3. Histology of the hearts from $crt^{+/-}$ and $crt^{-/-}$ embryos. A, B, and C show histological analysis of the 12.5-, 14.5-, and 18-d-old $crt^{+/-}$ embryos, respectively. A', B', and C' show histology of the 12.5-, 14.5-, and 18-d-old $crt^{-/-}$ embryos. $crt^{-/-}$ embryos show deep intertrabecular recesses and increased fenestration associated with the thinner ventricular wall. Sections were stained with hematoxylin and eosin as described in Materials and Methods.

Figure 4. Histological analysis of the heart of $crt^{+/-}$ and $crt^{-/-}$ 18-d-old embryos. Sections were stained with hematoxylin and eosin as described in Materials and Methods. The upper panel shows the apex of the heart in $crt^{+/-}$ and $crt^{-/-}$ embryos. The lower panel shows ventricular walls in $crt^{+/-}$ and $crt^{-/-}$ animals.

for the presence of the GFP transgene by PCR, followed by analysis of sections prepared from the transgenic and wild-type littermates.

Fig. 6, A and B, shows sagittal sections of a 9.5-d-old transgenic mouse embryo. The highest fluorescent signal, indicative of high expression of GFP, was found in the cardiovascular system including: ventricular walls, atrial walls, aortic sac, sinus venosus, dorsal aorta, and some of the smaller arteries (e.g., branchial arch arteries). This indicates that the highest activity of the calreticulin promoter occurs in these tissues at day 9.5 of embryonic development. The optic vesicle and the lining of brain ventricles also exhibited high calreticulin promoter activity (Fig. 6, A and B). At 10.5 d of embryonic development the calreticulin promoter remained highly active in cardiovascular and brain tissue, but its activity was also detected in the midgut and in intersomitic vessels (Fig. 6, C and D). In older embryos (13.5-d-old) high activity of the calreticulin promoter was maintained in the heart and arteries (Fig. 6 E). In addition, activation of the calreticulin promoter was also seen in the liver, midgut, and the umbilical hernia.

A high level of activation of the calreticulin promoter

continued in the cardiovascular system and umbilical hernia in the 14.5-d-old mouse embryo (Fig. 7 A). The expression of GFP reporter protein was localized exclusively to the cytosol of the myocytes of atria and ventricles (Fig. 7 B). There was no fluorescent signal in the thoracic wall and blood cells, indicating differential expression of GFP in these tissues. At late stages of development (18-d-old embryos) a relatively low fluorescent signal, indicative of a lower expression of GFP, was found in the heart (Fig. 7 C). A negligible level of fluorescence was found in the heart of 3-wk-old transgenic mice (Fig. 7 E). This indicates that the activity of the calreticulin promoter is downregulated at late stages of development and after birth. These findings are in agreement with earlier observations that mature hearts express a low level of calreticulin (Fliegel et al., 1989a,b; Milner et al., 1991; Tharin et al., 1996).

To confirm the expression of GFP protein was under the control of the calreticulin promoter in the cardiovascular system, we carried out immunohistological analysis of GFP transgenic embryos. Fig. 8 shows immunohistological staining of sagittal section of hearts from transgenic mice with antibodies to GFP. In agreement with observed fluorescent signals of GFP (Figs. 6 and 7) the protein was highly expressed in the embryonic heart (Fig. 8, C and D). There was no staining of the wild-type embryonic heart with anti-GFP antibodies (Fig. 8, A and B). To determine if activation of the calreticulin promoter correlated with expression of calreticulin protein we carried out immunohistological analysis of mouse embryos with anticalreticulin antibodies. Fig. 9 shows low (A–D) and high (A'–D') magnification of immunohistological staining of 9.5- (A, A'), 13.5- (B, B'), and 18- (C, C') d-old embryos and mature (D) hearts. Calreticulin protein was highly expressed in myocytes during early stages of embryonic development (Fig. 9, A', B', and C'). The highest expression of calreticulin was observed in the 13.5-d-old embryonic heart
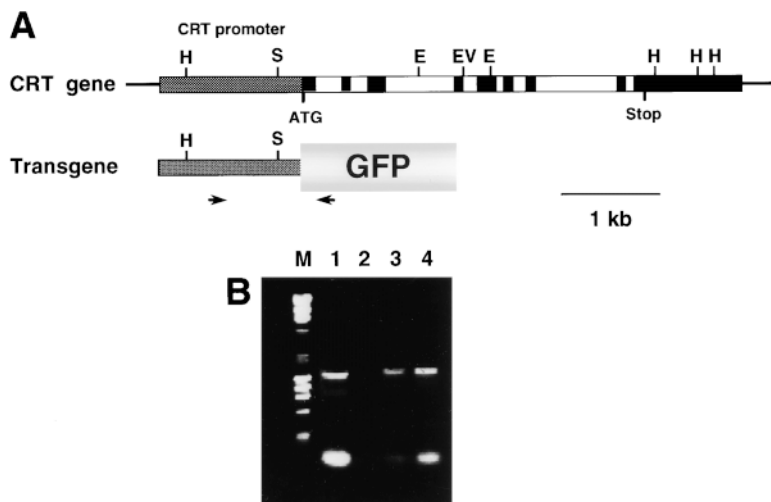
(Fig. 9 B'). In agreement with our earlier biochemical studies (Fliegel et al., 1989a,b; Milner et al., 1991; Tharin et al., 1996), virtually no staining for calreticulin was detected in the mature heart (Fig. 9 D'). This is in full agreement with the levels of GFP expressed in GFP transgenic mice (Figs. 6 and 7).

Nuclear Translocation of NF-AT3 Is Impaired in Calreticulin Knockout Cells

Recent observations indicate that the NF-AT3 transcription factor plays an important role in cardiac hypertrophy and development (de la Pompa et al., 1998; Molkentin et al., 1998; Ranger et al., 1998). The activation of NF-AT is controlled by calcineurin, a Ca^{2+} calmodulin-dependent phosphatase (Timmerman et al., 1996). Dephosphorylation of NF-AT by activated calcineurin triggers its nuclear translocation (Rao et al., 1997). To determine whether the role of calreticulin in cardiac development might be associated with NF-AT nuclear translocation, we isolated mouse embryonic fibroblasts from $crt^{-/-}$ and wild-type embryos and tested them for nuclear import of NF-AT3 transcription factor. Cells were stimulated with bradykinin to induce depletion of the intracellular Ca^{2+} stores (Hashii et al., 1993; Waser et al., 1997) and activation of calcineurin (Timmerman et al., 1996). Fig. 10 A shows that wild-type cells expressed calreticulin and the protein is localized to an ER-like network. As expected, there was no expression of calreticulin in $crt^{-/-}$ cells (Fig. 10 B). In wild-type cells stimulated with bradykinin, NF-AT3 was efficiently translocated to the nucleus (Fig. 10 A'). However, nuclear import of NF-AT3 in $crt^{-/-}$ cells was impaired and the majority of the transcription factor was found in the cytoplasm (Fig. 10 B') indicating that NF-AT3 transcription factor was not efficiently translocated into the nucleus. Nuclear translocation of NF-AT3 was reestablished in the $crt^{-/-}$ cells transiently transfected with calreticulin expression vector (Fig. 10, C and C').

InsP3-dependent Ca2+ Release Is Inhibited in Calreticulin-deficient Cells

It is well established that Ca^{2+} release by the $InsP_3$-depen-

dent pathway is required to activate calcineurin, leading to nuclear import of NF-AT transcription factor (Timmerman et al., 1996; Dolmetsch et al., 1997; Kehlenbach et al., 1998). To assess whether calreticulin-deficient cells exhibit any alteration in Ca^{2+} homeostasis, we used the fluorescent Ca^{2+} indicator fura-2. Wild-type and $crt^{-/-}$ mouse embryonic fibroblasts were stimulated with either thapsigargin, an inhibitor of SERCA type Ca^{2+} pumps (Thastrup et al., 1990), or with bradykinin, a potent activator of the $InsP_3$-dependent Ca^{2+} release channel located in the ER (Hashii et al., 1993). When cells were stimulated with thapsigargin, the peak amplitude and the duration of enhanced cytoplasmic Ca^{2+} concentration were comparable in wild-type and in $crt^{-/-}$ mouse embryonic fibroblasts (Fig. 11 A). Next we investigated the effects of bradykinin, a receptor agonist known to activate phospholipase C and $InsP_3$-dependent Ca^{2+} release from the ER (Hashii et al., 1993). Fig. 11 B (solid line) shows that bradykinin caused a rapid and transient increase in the cytoplasmic Ca^{2+} concentration in wild-type cells, but not in $crt^{-/-}$ mouse embryonic fibroblasts (Fig. 10 B, dotted line). Ca^{2+} release by $InsP_3$-dependent pathway was clearly impaired in calreticulin-deficient cells.

Discussion

In this study we demonstrated that disruption of the calreticulin gene results in embryonic lethality. Analysis of the $crt^{+/-}$ and $crt^{-/-}$ embryos showed defects in cardiac morphology. The $crt^{-/-}$ mice show a marked decrease in ventricular wall thickness and deep intertrabecular recesses in the ventricular walls. Investigation of the activation pattern of the calreticulin gene during embryonic development revealed that at early stages of embryogenesis the highest level of activation of the calreticulin gene was in the cardiovascular system: ventricular walls, atrial walls, aortic sac, sinus venosus, dorsal aorta, and some of the smaller arteries. In later stages of embryogenesis a high activity of the promoter in the cardiovascular system was maintained, but activation of the gene was also seen in the brain, midgut, intersomitic vessels, and, finally, in the liver
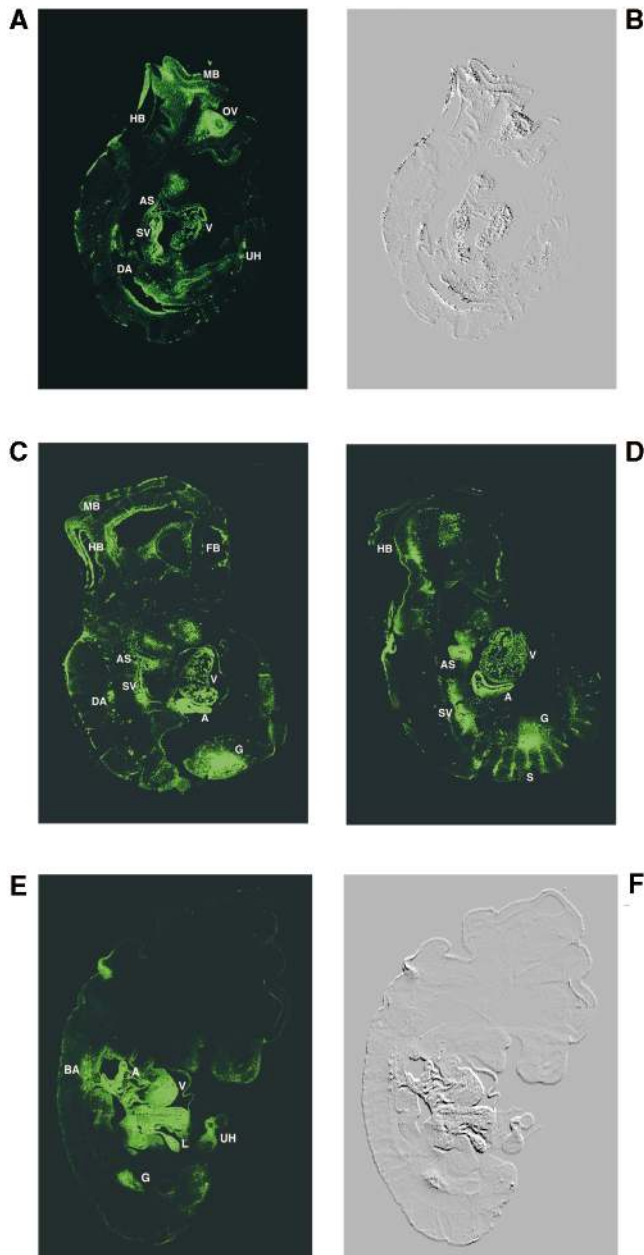
Figure 6. Developmental activation of calreticulin promoter. (A and B) Sagittal section of a 9.5-d-old mouse embryo expressing the GFP reporter protein under the control of the calreticulin promoter. (B) A phase-contrast of the image seen in A. (C and D) Sagittal sections of 10.5-d-old mouse embryos from GFP transgenic mice. (C) The first section shows high expression of GFP in the atria (A) and ventricles (V). Fluorescent signals are seen mainly in the cardiovascular and the nervous systems. (D) Additional sections of the embryo shown in C indicate high fluorescence in the intersomitic vessels (S). (E and F) Analysis of 13.5-d-old transgenic mouse embryo. (E) Confocal image showing the fluorescent signal in the cardiovascular system, liver, gut, and umbilical hernia. (F) A phase-contrast image of E. Tissues expressing GFP show a highly fluorescent signal: A, atria; AS, aortic sac; BA, branchial arteries and branches from the pulmonary arteries and arches of the aorta; DA, dorsal aorta; FB, fore brain; G, gut; HB, hind brain; L, liver; MB, mid brain; MG, midgut; OV, optic vesicle; SV, sinus venosus; S, somite; UH, umbilical hernia; and V, ventricles.
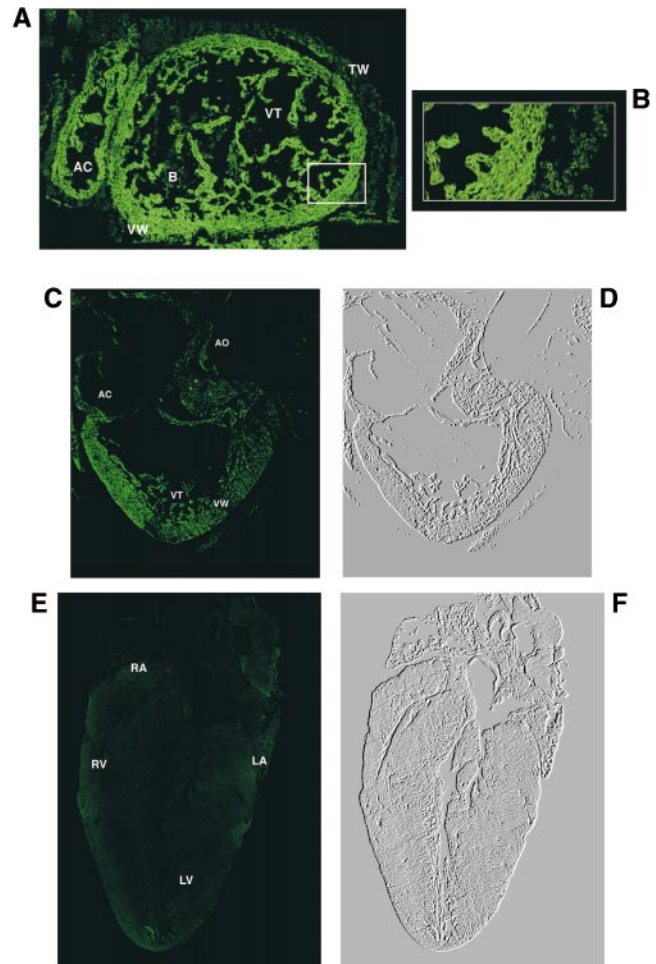
Figure 7. Confocal image of the hearts at different stages of development. (A and B) Cross-sections of the heart from 14.5-d-old embryo. GFP expression is localized to both atrial and ventricular walls but the fluorescent signal is also seen in the atrial and ventricular trabeculae. (B) The highest fluorescent signal found in the cardiomyocytes is shown. (C) GFP expression in the heart of 18-d-old embryo. (D) A phase-contrast image of C. (E) A negligible level of expression of GFP in the heart of 3-wk-old mouse is shown. (F) A phase-contrast image of E. Symbols: AC, atrial chamber; B, blood cells; TW, thoracic wall; VT, ventricular trabeculae; and VW, ventricular wall.

and the umbilical hernia. Analysis of InsP$_3$-dependent Ca^{2+} release and of nuclear import of NF-AT3 transcription factor in cells isolated from $crt^{-/-}$ and wild-type mice revealed that calreticulin is a component of the Ca^{2+} regulatory system in these cells. Therefore, calreticulin influences the Ca^{2+} and calcineurin-dependent pathway in developing heart.

In the adult, calreticulin is expressed mainly in nonmuscle and smooth muscle cells, and is only a minor component of the skeletal muscle and cardiac sarcoplasmic reticulum (Fliegel et al., 1989a,b; Milner et al., 1991; Tharin et al., 1996). However, data presented in Figs. 6–9 show that calreticulin is highly expressed in the cardiovascular system during early embryogenesis. Thus, it was not surpris-
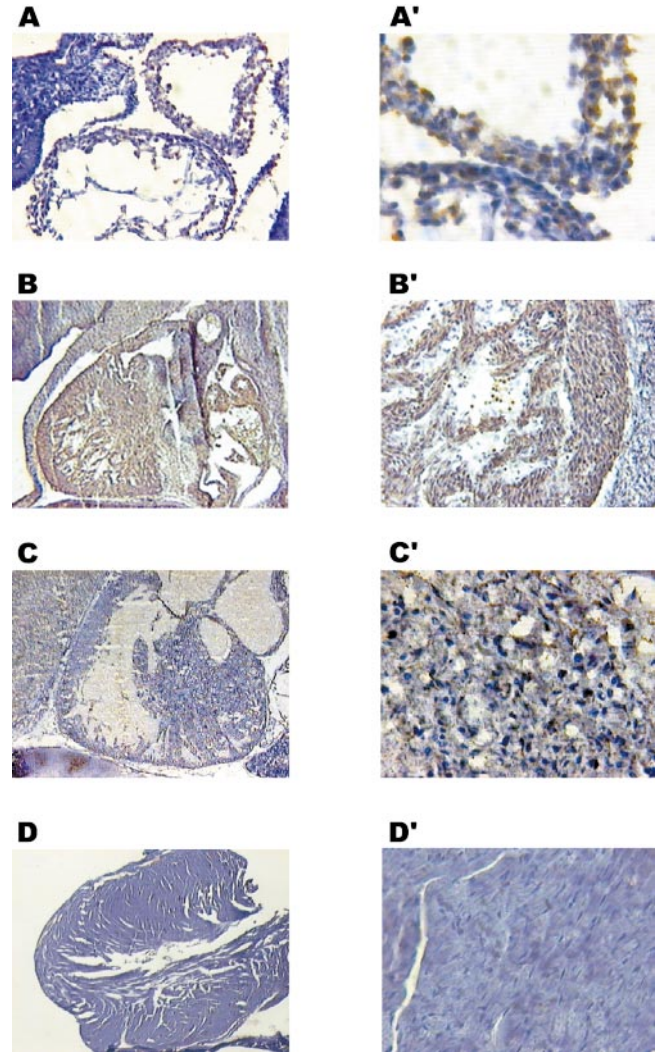
A. Control **B. Control**

C. GFP **D. GFP**
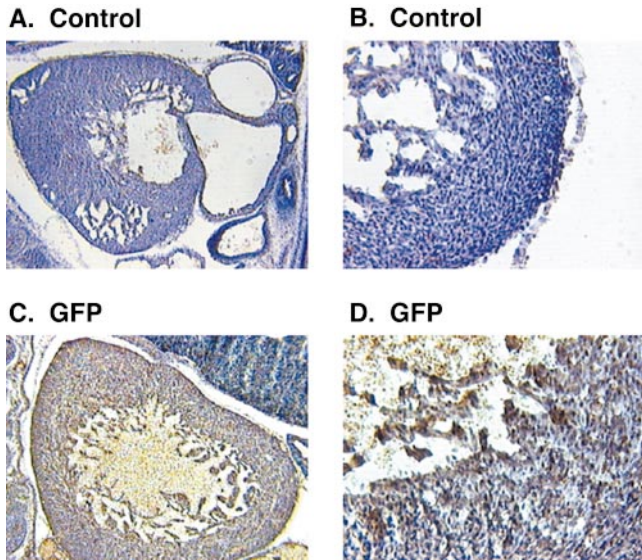
Figure 8. Immunohistochemical analysis of the GFP transgenic mice. Sagittal sections of 13.5-d-old mouse embryos were stained with specific antibodies followed by DAB (brown color). The sections were counter-stained with hematoxylin to visualize the nuclei. Low (A) and high magnification (B) images from cryostat sections of a wild-type mouse embryo stained with anti-GFP antibody. Low (C) and high (D) magnification images from GFP transgenic mouse showing the GFP positive staining in the ventricular myocytes.

A **A'**

B **B'**

C **C'**

D **D'**

Figure 9. Immunohistochemical analysis of the transgenic mice with anticalreticulin antibodies. Sagittal sections of 9.5- (A), 13.5- (B), and 18- (C) d-old embryos and mature (D) mouse hearts were stained with anticalreticulin antibodies followed by DAB (brown color). High magnification picture (A'–D') shows that calreticulin protein was highly expressed in myocytes during early stages of embryonic development (A', B', and C'). No significant staining for calreticulin was detected in the mature heart (D'). The sections were counterstained with hematoxylin.

ing to find that $crt^{-/-}$ mice have defects in cardiac development and function. The only other gross morphological change in $crt^{-/-}$ embryos was the failure to absorb the umbilical hernia. It is unlikely that embryonic lethality of calreticulin-deficient mice occurs because of failure to absorb the umbilical hernia since this pathology is not embryonic lethal in humans (Byrne, 1991). Embryonic lethality of $crt^{-/-}$ mice most likely resulted from a lesion in cardiac development.

Numerous functions have been postulated for calreticulin including: modulation of steroid-mediated gene expression (Burns et al., 1994; Dedhar et al., 1994; Michalak et al., 1996), chaperone activity (Helenius et al., 1997), regulation of cell adhesion (Coppolino et al., 1995, 1997; Opas et al., 1996), and regulation of Ca^{2+} homeostasis (Liu et al., 1994; Bastianutto et al., 1995; Camacho and Lechleiter, 1995; Mery et al., 1996; Coppolino et al., 1997; Fasolato et al., 1998; John et al., 1998). A loss of any of these potential functions of calreticulin could lead to a lesion in cardiac development. Because there were no obvious histological abnormalities in any other tissues during embryogenesis, it is unlikely that this is directly due to chaperone function of calreticulin, or its role in the regulation of cell adhesion.

Cardiac development is an extremely complex process under strict transcriptional control (Rossant, 1996; Fishman and Chien, 1997; Sucov, 1998; Creazzo et al., 1998). The first morphogenetic event in mouse cardiac development is the formation of a primordial heart tube by cells that segregate from the splanchnic mesoderm in 7-d-old e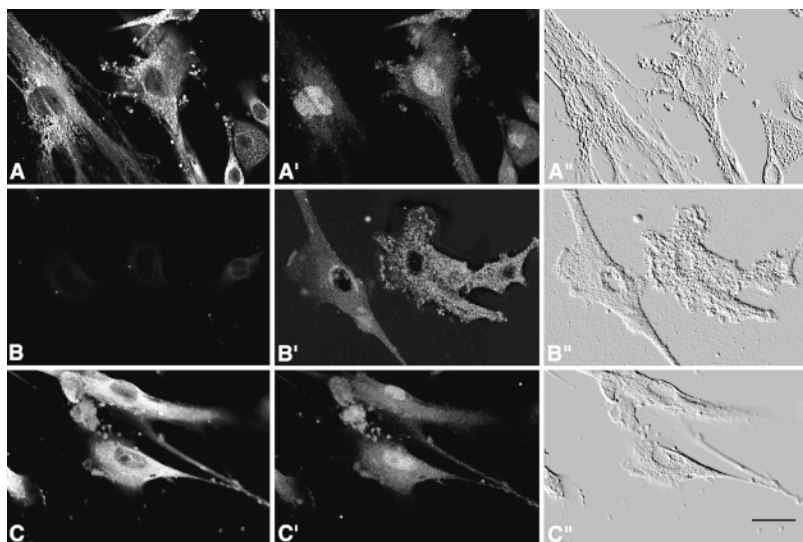mbryos (DeRuiter et al., 1992). Between 8 and 9.5 d, the primitive heart tube begins to exhibit rhythmic contractions and undergoes asymmetrical elongation, creating an S-shape loop (DeRuiter et al., 1992). Later in embryogenesis, the growing heart progresses through several additional morphological stages including all of the following: the formation of endocardial cushion tissue, septation, trabeculation, compaction (expansion of the ventricular wall), and envelopment of the epicardial mantle (DeRuiter et al., 1992; Mikawa and Fischman, 1996). Based on analogies to skeletal myogenesis, it is presumed that cardiomyocyte lineage commitment is directed by a group of tissue-specific transcription factors (Olson and Srivastova, 1996).

However, the factors essential for cardiomyocyte differentiation in vertebrates remain poorly characterized (Ol-
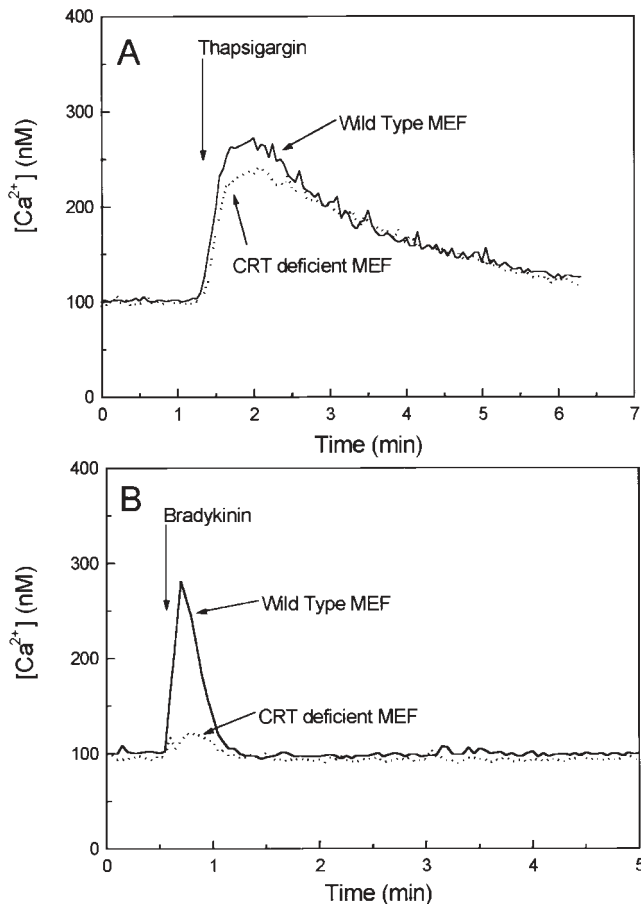
son and Srivastova, 1996). How can calreticulin, an ER lumenal protein, affect cardiac development? In this work we have shown that nuclear import of NF-AT3 transcription factor is impaired in *crt*$^{-/-}$ cells indicating that calreticulin plays a role in the Ca^{2+}/calcineurin/NF-AT/GATA-4 transcription pathway (Molkentin et al., 1998). Cardiomyogenesis depends on activation of the GATA family of transcription factors (Grepin et al., 1994; Evans, 1997). GATA-4 null mice display a severe defect in the formation of the cardiac tube, which is required for the migration and folding of the precardiogenic splanchnic mesoderm (Kuo et al., 1997; Molkentin et al., 1997). Furthermore, inhibition of GATA-4 expression affects terminal cardiomyocyte differentiation (Grepin et al., 1994). During cardiomyogenesis NF-AT, in the nucleus, forms active heterodimer complexes with GATA-4 and significantly enhances its transcriptional activity (Molkentin et al., 1998). The ability of NF-AT to translocate to the nucleus depends on the activation of calcineurin phosphatase activity and efficient dephosphorylation of the transcription factor (Rao et al., 1997). Therefore, it is not surprising that NF-AT–deficient mice are not viable and the major defect in these animals relates to cardiac development (de la Pompa et al., 1998; Ranger et al., 1998).

Activation of calcineurin and nuclear import of NF-AT requires Ca^{2+} release by the InsP$_3$-dependent pathway (Timmerman et al., 1996; Dolmetsch et al., 1997). In this study we show that calreticulin-deficient mouse embryonic fibroblasts do not have a measurable InsP$_3$-dependent Ca^{2+} release when stimulated with bradykinin. This is in agreement with a previous report in which the Ca^{2+} response to bradykinin was diminished as a result of antisense oligodeoxynucleotide downregulation of calreticulin expression (Liu et al., 1994). Calreticulin is a Ca^{2+}-binding protein that affects Ca^{2+} storage in the ER lumen (Bastianutto et al., 1995; Mery et al., 1996; Fasolato et al., 1998). It may regulate function of the ER Ca^{2+}-release channel (InsP$_3$ receptor), Ca^{2+}-ATPase (SERCA2b) (Camacho and Lechleiter, 1995; John et al., 1998), and store-operated Ca^{2+} influx (Mery et al., 1996). Camacho and

Lechleiter (1995) proposed that calreticulin may be responsible for modulation of the Ca^{2+} release function of the InsP$_3$ receptor and/or of the Ca^{2+} transport function of SERCA. Recently, John et al. (1998) reported that calreticulin may interact with SERCA2b, resulting in a lower transport capacity by the Ca^{2+}-ATPase. Here we show that loss of calreticulin reduces Ca^{2+} release through the InsP$_3$ receptor. Despite inhibition of the InsP$_3$-dependent Ca^{2+} release pathway, cardiomyocytes cultured from 14.5-d-old *crt*$^{-/-}$ embryos undergo spontaneous contraction indicating that their excitation-contraction coupling is functional in these cells (Mesaeli, N., and M. Michalak, unpublished observations). This implies that Ca^{2+} handling by the ER, but not by the cardiac sarcoplasmic reticulum, is affected in calreticulin knockout cardiomyocytes. ER and sarcoplasmic reticulum membranes may form separate functional entities in muscle cells (Tharin et al., 1996).

An intriguing finding of this work is that calreticulin-deficient mice develop omphalocele (umbilical hernia). At the early stages of embryogenesis the small intestine develops outside the body to form a physiological umbilical hernia. However, in 15.5-d-old embryos the intestine enters the body cavity and the body wall closes (only the umbilical cord and its contents remain in this region) (Kaufman, 1992). Failure of the intestine to return to the coelom results in an omphalocele (Byrne, 1991).

Recent studies indicate that umbilical hernia is associated with the function of a cyclin-dependent kinase inhibitory protein p57^{KIP2}, a regulator of cell proliferation (Eggenschwiler et al., 1997; Zhang et al., 1997). IGF-II receptor mutation and overexpression of IGF-II also results in omphalocele (Eggenschwiler et al., 1997). Disruption of the MARCKS gene that encodes a substrate of protein kinase C also leads to an omphalocele (Stumpo et al., 1995; Chen et al., 1996). Protein kinase C is activated by Ca^{2+}, suggesting that Ca^{2+} release from the ER may play an important role in the closure of umbilical hernia. GATA-4 transcription factor is also expressed during gut development by playing a role in the regulation of intestine epithelial cell differentiation (Gao et al., 1998). It is conceivable
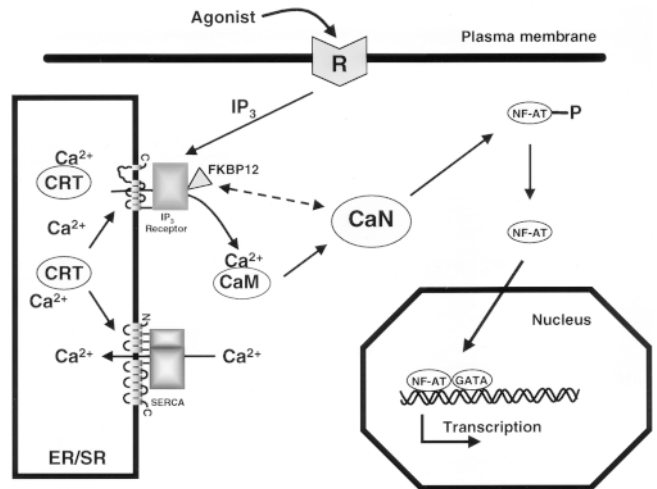
Figure 12. A model for the proposed role of calreticulin in the regulation of cardiac development. Myogenic signal from extracellular space activates the production of IP_3 that results in the release of Ca^{2+} from ER under the regulation of calreticulin (CRT). Increased intracellular Ca^{2+} binds to calmodulin (CaM) and activates calcineurin (CaN). CaN dephosphorylates NF-AT that translocates to the nucleus. In the nucleus NF-AT forms complexes with the GATA-4 transcription factors leading to activation of transcription of genes essential for cardiac development (Molkentin et al., 1998).

Figure 11. Bradykinin-induced elevation of cytoplasmic Ca^{2+} concentration is inhibited in $crt^{-/-}$ cells. Wild-type and $crt^{-/-}$ mouse embryonic fibroblasts (MEF) were loaded with the fluorescent Ca^{2+} indicator fura-2. Cells were stimulated with 1 μM thapsigargin, a Ca^{2+}-ATPase inhibitor (A), or with 200 nM bradykinin (B). Solid line shows wild-type mouse embryonic fibroblasts and the dotted line shows calreticulin (CRT)-deficient mouse embryonic fibroblasts.

that a similar Ca^{2+}/calcineurin/NF-AT/GATA-4 transcription pathway may be activated during cardiac development and closure of umbilical hernia. This may explain why NF-AT3–deficient mice also show signs of abdominal necrosis (de la Pompa et al., 1998).

In Fig. 12 we present a model in which calreticulin is linked to NF-AT function and cardiac development. Ca^{2+} release through the InsP$_3$-dependent pathway is required to activate calcineurin and to maintain NF-AT transcription factor in the nucleus (Timmerman et al., 1996; Dolmetsch et al., 1997). We propose that calreticulin regulates calcineurin activity indirectly by affecting Ca^{2+} release from the ER and the ability of NF-AT to translocate to the nucleus (Fig. 12). This model is supported by our demonstration that InsP$_3$-dependent Ca^{2+} release is inhibited in calreticulin-deficient cells, concomitant with the inhibition of the NF-AT nuclear import.

The role of the InsP$_3$ receptor and the InsP$_3$-dependent pathway in cardiac development is further supported by studies on another regulator of the InsP$_3$ receptor and

Ca^{2+} release, the immunophilin protein FKBP12 (FK506 binding protein) (Brillantes et al., 1994; Cameron et al., 1995). Knockout of the FKBP12 gene is also embryonic lethal and the animals show cardiac defects (Shou et al., 1998). FKBP12 could be considered as a cytosolic regulator of the ER Ca^{2+} release channel, whereas calreticulin could be a lumenal regulator of Ca^{2+} homeostasis. The calreticulin/Ca^{2+}/calcineurin/NF-AT/GATA-4 regulatory pathway is critical for the developing heart (Molkentin et al., 1998), but it is likely to be switched off in the adult myocardium and many components are absent or present at very low levels (NF-AT, GATA-4, and calreticulin) in the mature cardiomyocytes (de la Pompa et al., 1998; Fliegel et al., 1989a,b; Molkentin et al., 1998, and the references therein). In mature organisms NF-AT and calreticulin may play a more important role in the immune response (Burns et al., 1992; Dupuis et al., 1993; Rao et al., 1997; Andrin et al., 1998). Our results demonstrate an essential role for calreticulin during embryogenesis. Although calreticulin is not a transcription factor, this work shows that the protein is a regulator of Ca^{2+} homeostasis and of transcriptional pathways involved in cardiac development.

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