Cardiac-specific Expression of Calcineurin Reverses Embryonic Lethality in Calreticulin-deficient Mouse*

Received for publication, September 26, 2002, and in revised form, October 9, 2002
Published, JBC Papers in Press, October 10, 2002, DOI 10.1074/jbc.M209900200

Lei Guo§§, Kimitoshi Nakamura‡, Jeffery Lynch‡, Michal Opas**, Eric N. Olson‡‡, Luis B. Agellon§§§, and Marek Michalak‡‡‡
From the §§Canadian Institutes of Health Research Membrane Protein Research Group, §§Canadian Institutes of Health Research Molecular and Cell Biology of Lipids Research Group, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, ‡‡‡Department of Pathology and Laboratory Medicine, University of Toronto, Toronto, Ontario M5S 1A8, Canada, and the ‡‡Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9148

Calreticulin is an endoplasmic reticulum resident Ca\(^{2+}\)-binding chaperone. The importance of the protein is illustrated by embryonic lethality because of impaired cardiac development in calreticulin-deficient mice. The molecular details underlying this phenotype are not understood. In this study, we show that overexpression of activated calcineurin reverses the defect in cardiac development observed in calreticulin-deficient mice and rescues them from embryonic lethality. The surviving mice show no defect in cardiac development but exhibited growth retardation, hypoglycemia, increased levels of serum triacylglycerols, and cholesterol. Reversal of embryonic lethality because of calreticulin deficiency by activated calcineurin underscores the impact of the calreticulin-calcineurin functions on the Ca\(^{2+}\)-dependent signaling cascade during early cardiac development. These findings show that calreticulin and calcineurin play fundamental roles in Ca\(^{2+}\)-dependent pathways essential for normal cardiac development and explain the molecular basis for the rescue of calreticulin-deficient phenotype.

Calreticulin is an endoplasmic reticulum resident Ca\(^{2+}\)-binding protein that affects endoplasmic reticulum Ca\(^{2+}\) and participates in the folding of proteins (1). Calreticulin deficiency in mice is embryonically lethal because of impaired cardiac development (2, 3). Mice homozygous for the calreticulin gene disruption showed a marked decrease in ventricular wall thickness and deep intertrabecular recesses in the ventricular walls (2). The molecular details underlying this phenotype are not understood. However, in the absence of calreticulin, cells have markedly impaired inositol 1,4,5-trisphosphate (IP\(_3\))-dependent Ca\(^{2+}\) release, which prohibits the nuclear translocation of NF-AT in response to Ca\(^{2+}\) stimulation (2), resulting in the impaired regulation of NF-AT-dependent genes. Calcineurin phosphatase activity is required for nuclear translocation of NF-AT (4, 5). Calcineurin is a serine/threonine phosphatase, which consists of a catalytic A subunit and a regulatory B subunit (5). A Ca\(^{2+}\)- and calcineurin B-dependent regulatory region is located in the C-terminal 200 amino acids of the calcineurin A subunit (5).

Sustained Ca\(^{2+}\) release from the endoplasmic reticulum and Ca\(^{2+}\) influx via the plasma membrane are required to fully activate calcineurin phosphatase activity (5, 6). Calreticulin-deficient cells have impaired IP\(_3\)-dependent Ca\(^{2+}\) release (7) and inhibited nuclear translocation of NF-AT (2), indicating that calcineurin activity is impaired in the absence of calreticulin. Both calreticulin and calcineurin have been implicated in cardiac development and hypertrophy (2, 8). The elimination of calreticulin in mice causes death in utero because of defective cardiac development (2), whereas increased activity of calcineurin leads to the development of cardiac hypertrophy (8). These observations indicate that Ca\(^{2+}\) and calcineurin-dependent signaling pathways in the heart may underlie the embryonic lethality displayed by calreticulin-deficient mice. Here we tested this hypothesis and reconstituted the hearts of calreticulin-deficient mice with a truncated constitutively active form of calcineurin to reestablish the Ca\(^{2+}\)-dependent cascade downstream of calreticulin. We show that the overexpression of activated calcineurin reverses the defect in cardiac development observed in calreticulin-deficient mice and rescues them from embryonic lethality.

EXPERIMENTAL PROCEDURES

Transgenic Animals and Histological Analysis—Mice with cardiac-specific expression of activated calcineurin (amino acid residues 1–389) were created using the α-MHC promoter (8). These animals have been extensively characterized (8). Homozygote transgenic mice with respect to the activated calcineurin transgene were used in all experiments. The creation of calreticulin-deficient mice was described previously (2). Mice bearing the activated calcineurin transgene (FVB/N strain) were crossed with crt\(^{-/-}\) (C57BL/6j strain) mice. The progeny of this cross were interbred to generate calreticulin-deficient mice expressing activated calcineurin in the heart. The breeding of the transgenic animals was carried out the University of Alberta Health Sciences Transgenic Facility. Histological analysis of cardiac tissue was carried out as described previously (2). Sections were stained with hematoxylin and eosin.

Calcinurin-dependent Translocation of NF-ATc1—cDNA encoding NF-ATc1 was inserted into the BamH1 site of pEGFP-C1 (Clontech) to

calreticulin; CaN, calcineurin; E, embryonic day; GFP, green fluorescent protein; α-MHC, myosin heavy chain; NF-AT, nuclear factor of activated T-cells.

*This work was supported by the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Ontario. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. ‡Postdoctoral fellow of the Heart and Stroke Foundation of Canada.
¶¶Both authors contributed equally to this work.
*Recipient of a Studentship from the Heart and Stroke Foundation of Canada and the Alberta Heritage Foundation for Medical Research.
*Alberta Heritage Foundation for Medical Research Senior Scholar. 
\| Canadian Institutes of Health Research Senior Investigator and a Medical Scientist of the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed: Dept. of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-2256; Fax: 780-492-0886; E-mail: Marek.Michalak@ualberta.ca.
**The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; crt, calreticulin; CaN, calcineurin; E, embryonic day; GFP, green fluorescent protein; α-MHC, myosin heavy chain; NF-AT, nuclear factor of activated T-cells.
Calreticulin-deficient Mouse

**Fig. 1.** Calcinurin-dependent nuclear translocation of NF-ATc1 in calreticulin-deficient cells. Fibroblasts isolated from calreticulin-deficient and wild type mice were transiently transfected with GFP-NF-ATc1 and the activated calcineurin expression vectors. Localization of GFP-NF-ATc1 was monitored using a Zeiss Axiovert S100 fluorescent microscope with an excitation wavelength of 490 nm and emission wavelength of 520 nm. GFP-NF-ATc1 translocates to the nucleus in the presence of the activated calcineurin. wt, wild type. Bar, 25 μm.

**Fig. 2.** Histological and immunological analysis of the hearts from the calreticulin-deficient embryos. A, Western blot analysis of proteins from day 17.5 embryonic hearts probed with anti-calcineurin antibodies. Cardiac tissue was isolated from the embryos and homogenized in a buffer containing protease inhibitors (16). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-calcineurin (anti-CaN) antibodies. Lane 1, wild type; lane 2, crt−/−; lane 3, crt−/−:activated CaN; lane 4, crt−/−:activated CaN. Upper protein band corresponds to the endogenous CaN, and the lower band represents truncated activated CaN protein. B, sections of day 17.5 embryonic hearts were stained with hematoxylin and eosin. The panels 1 and 2 show a longitudinal section of the heart from a wild type (crt+/−) and a crt−/− embryo, respectively. Panels 3 and 4 show histology of hearts from crt−/−:activated CaN and crt−/−:activated CaN embryos, respectively. Hearts in crt−/− exhibit intertrabecular recesses and thin ventricular wall (2, 3). In contrast, crt−/−:activated CaN and crt−/−:activated CaN have hypertrophied ventricular wall. Bar, 500 μm.

**Fig. 3.** Calcineurin rescues calreticulin-deficient phenotype. Upper panel, a photograph of calreticulin-deficient mouse rescued with the activated calcineurin (crt−/−:activated CaN) and its sibling (crt−/−:activated CaN) at 4 weeks of age. Both animals express the activated calcineurin in the heart. Lower panel, cardiac tissue was isolated from crt−/−:activated CaN and crt−/−:activated CaN and homogenized in a buffer containing protease inhibitors (16). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-calcineurin (anti-CaN) or anti-calreticulin (anti-CRT) antibodies. Lane 1, crt−/−:activated CaN; lane 2, crt−/−:activated CaN. Upper protein band corresponds to the endogenous CaN, and the lower band represents truncated, activated CaN protein.

**TABLE I**

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of littermates</th>
<th>crt−/+</th>
<th>crt−/−</th>
<th>Upper protein band</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14.5</td>
<td>45</td>
<td>84 (25%)</td>
<td>162 (49%)</td>
<td>77 (24%)</td>
</tr>
<tr>
<td>E17.5</td>
<td>20</td>
<td>36 (24%)</td>
<td>80 (54%)</td>
<td>34 (23%)</td>
</tr>
</tbody>
</table>

Cardiac-specific expression of activated calcineurin was driven by the α-MHC promoter (8). The progeny of this cross was interbred with crt−/− males and crt−/− females to produce calcinurin transgenic mice that were heterozygous for the inactive calreticulin allele and homozygous for activated calcineurin (designated crt−/−:activated-CaN). Male and female crt−/−:activated-CaN were crossed and displayed the expected Mendelian ratios of crt−/−:activated-CaN, crt−/−:activated-CaN, and crt−/−:activated-CaN mice. crt−/−, crt−/−, and crt−/− designate crt−/−:activated-CaN, crt−/−:activated-CaN, and crt−/−:activated-CaN mice, respectively.

The activation of calcineurin normally requires a sustained Ca2+ release by the IP3-dependent pathway (13), but the dele-

generate a GFP-NF-ATc1. SV40 immortalized (9) crt−/− and crt−/− fibroblasts were transiently transfected with the GFP-NF-ATc1 expression vector using Effectene Transfection reagent (Qiagen). The cells were also transfected with pcDNA3.1 plasmids containing cDNA encoding constitutively active calcineurin (activated calcineurin) (10). As a control, cells were transfected with a pEGFP-C1 with cDNA encoding NF-ATc1 cloned in non-sense orientation. Cells were grown on 25-mm circular coverslips (Fisher Scientific) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Images were captured with a Zeiss Axiovert S100 fluorescent microscope using an excitation wavelength of 490 nm and emission wavelength of 520 nm. All of the pictures were pseudo-colored using Northern Eclipse 5.0 software.

**Blood Glucose and Serum Lipid Determination**—Blood was collected and glucose was measured using Glucometer Elite Blood Glucose Meter (model 3905E, Bayer) with Glucometer Elite blood glucose test strips with 1.1–3.3 mmol/liter sensitivity. Serum total cholesterol and triacylglycerol concentrations were measured using a commercial diagnostic assay (kit number 352 for total cholesterol and kit number 339 for triacylglycerols, respectively, from Sigma).

**Miscellaneous**—Proteins were separated by SDS-PAGE on 10% polyacrylamide gels as described by Laemmli (11), transferred to nitrocellulose membranes, and stained by immunoblotting with affinity-purified rabbit anti-calreticulin or anti-calcineurin antibodies (12). Genomic DNA was isolated from mouse tissue, and the presence of the disrupted calreticulin gene was verified either by Southern blotting or PCR (2). The calreticulin transgene was detected by PCR using the 5′ primer 5′-TATCTCCCCCATAGAGTTT-3′ (α-MHC promoter region) and the 3′ primer 5′-TGATCCCCACCTCAAAACAACCTCA-3′ (calcinurin-coding region) using Pfu polymerase. Southern blot analysis was also carried out using the XhoI-SsII (480 bp) DNA fragment derived from pBS-α-MHC calreticulin plasmid (originally used to generate the transgenic mice) as a probe.

**RESULTS AND DISCUSSION**

The activation of calcineurin normally requires a sustained Ca2+ release by the IP3-dependent pathway (13), but the dele-
Calreticulin-deficient Mouse

Fig. 4. Normal cardiac development in crt<sup>−/−</sup> embryos expressing the activated-calcinurin in the heart. Histological analysis of hearts for 4-week-old crt<sup>−/−</sup>:activated CaN and crt<sup>−/−</sup>:activated CaN mice. Histological sections revealed that a low level of endogenous calcineurin was expressed in the heart. Microscopic analysis of hematoxylin- and eosin-stained sections demonstrated comparable ventricular wall thickness and myofibrillar organization. Bar, 30 μm.

Fig. 5. Blood sugar and lipid analysis in calreticulin-deficient mice. Blood sugar and lipid analysis was carried out as described under "Experimental Procedures." Calreticulin-deficient mice were hypoglycemic and accumulated large quantities of cholesterol and triacylglycerols in their serum. Open bars, serum from crt<sup>−/−</sup>:activated CaN mice. Hatched bars, values for a serum isolated from two different crt<sup>−/−</sup>:activated CaN mice. Data for crt<sup>−/−</sup>:activated CaN mice are the means ± S.D. for nine independent experiments.

Fig. 6. A model for the proposed relationship between calreticulin and calcineurin during cardiac development. Signals from the extracellular space activate the production of IP<sub>3</sub>, leading to release of Ca<sup>2+</sup> from the endoplasmic reticulum (ER) and activation of calcineurin-dependent processes (left panel). In the absence of calreticulin (middle panel), there is no Ca<sup>2+</sup> release from the ER; therefore endogenous calcineurin remains inactive, resulting in the inhibition or significant reduction of calcineurin-dependent processes during cardiac development. Activated calcineurin rescues calreticulin-deficient embryonic lethality because activated calcinurin is fully active independent of Ca<sup>2+</sup> signal (right panel).
was a >15-fold increased expression of activated calcineurin when compared with the endogenous protein. The expression of activated calcineurin was also significantly increased in comparison with expression of the protein in E17.5 embryonic heart.

At birth, calreticulin-deficient mice (crt⁻/⁻:activated CaN) were overtly indistinguishable from their wild-type (crt⁺/⁺: activated CaN) and heterozygous (crt⁺/-:activated CaN) littermates. However, all of the homozygote calreticulin knock-out mice (crt⁻/⁻:activated CaN) showed significant growth retardation after the first week of life (Fig. 3) and died 3–5 weeks after birth. Importantly, histological analysis did not show any significant differences in cardiac morphology among these mice (Fig. 4). Cardiac hypertrophy was noted in these mice. Of the five mature heart. High calcineurin phosphatase activity in postnatal hearts leads to hypertrophy and heart failure (8), and calcineurin-dependent pathways are detrimental to normal function of the heart. The underlying cause for these metabolic aberrations is not yet clear, but the results suggest the importance of calreticulin (17). Here we show that the expression of activated calcineurin, a Ca<sup>2+</sup>- and calmodulin-dependent phosphatase, reverses embryonic lethality in calreticulin-deficient mice. Fig. 6 shows a putative model describing the relationship between cardiac development in the endoplasmic reticulum lumen and calcineurin in the cytoplasm during cardiac development. Under normal physiological conditions, calreticulin is an important Ca<sup>2+</sup> storage chaperone in the lumen of the endoplasmic reticulum and is required for agonist-induced IP<sub>3</sub>-dependent Ca<sup>2+</sup> release from the endoplasmic reticulum (2, 7). Sustained Ca<sup>2+</sup> release from the endoplasmic reticulum is required for the activation of calcineurin and subsequent activation of calcineurin-dependent pathways including transcriptional processes (Fig. 6, left panel). In the absence of calreticulin, IP<sub>3</sub>-dependent Ca<sup>2+</sup> release from the endoplasmic reticulum in response to agonist is severely impaired (2, 7). Consequently, the endogenous calcineurin may not be fully activated, resulting in the failure to stimulate key Ca<sup>2+</sup> and calcineurin-dependent pathways critical for cardiac development (Fig. 6, middle panel). In crt⁻/⁻ mice, this defect in Ca<sup>2+</sup>-signaling manifests in impaired cardiac development, leading to embryonic lethality (2). Our findings suggest that the expression of activated calcineurin bypasses the requirement for agonist-induced InsP<sub>3</sub>-dependent Ca<sup>2+</sup> release (2, 7), thereby allowing activation of calcineurin-dependent pathways to occur in the absence of calreticulin (Fig. 6, right panel). The importance of calreticulin in Ca<sup>2+</sup> homeostasis as well as in protein folding and quality control are generally accepted (1). Here we have demonstrated that calreticulin-dependent pathways are the molecular basis for the rescue of calreticulin-deficient phenotype in mice. Calreticulin is an integral and essential component of cellular Ca<sup>2+</sup> homeostasis, and it may be a key upstream regulator of calcineurin function in the Ca<sup>2+</sup>-signaling cascade.

Acknowledgments—We thank Michel Puceat (CNRS Montpellier) for help during the preparation of this paper. We thank S. Aldred, M. Dabrowska, and E. Dziak for superb technical help.

REFERENCES

Cardiac-specific Expression of Calcineurin Reverses Embryonic Lethality in Calreticulin-deficient Mouse
Lei Guo, Kimitoshi Nakamura, Jeffery Lynch, Michal Opas, Eric N. Olson, Luis B. Agellon and Marek Michalak

doi: 10.1074/jbc.M209900200 originally published online October 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209900200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 17 references, 9 of which can be accessed free at http://www.jbc.org/content/277/52/50776.full.html#ref-list-1