On the Physiological Importance of Endoproteolysis of CAAAX Proteins

HEART-SPECIFIC RCE1 KNOCKOUT MICE DEVELOP A LETHAL CARDIOMYOPATHY*

Received for publication, September 10, 2003, and in revised form, November 14, 2003
Published, JBC Papers in Press, November 18, 2003, DOI 10.1074/jbc.M310081200

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Proteins terminating with a CAAAX motif, such as the Ras proteins and the nuclear lamins, undergo post-translational modification of a C-terminal cysteine with an isoprenyl lipid via a process called protein prenylation. After prenylation, the last three residues of CAAAX proteins are clipped off by Rce1, an integral membrane endoprotease of the endoplasmic reticulum. Prenylation is crucial to the function of many CAAAX proteins, but the physiologic significance of endoproteolytic processing has remained obscure. To address this issue, we used Cre/loxP recombination techniques to create mice lacking Rce1 in the heart, an organ where Rce1 is expressed at particularly high levels. The hearts from heart-specific Rce1 knockout mice manifested reduced levels of both the Rce1 mRNA and CAAX endoprotease activity, and the hearts manifested an accumulation of CAAX protein substrates. The heart-specific Rce1 knockout mice initially appeared healthy but died starting at 3–5 months of age. By 10 months of age, ~70% of the mice had died. Pathological studies revealed that the heart-specific Rce1 knockout mice had a dilated cardiomyopathy. By contrast, liver-specific Rce1 knockout mice appeared healthy, had normal transaminase levels, and had normal liver histology. These studies indicate that the endoproteolytic processing of CAAX proteins is essential for cardiac function but is less important for the liver.

Proteins terminating with a CAAAX motif, such as the nuclear lamins and the Ras and Rho proteins, undergo three sequential post-translational processing steps (1–4). First, the C-terminal cysteine (i.e. the C of the CAAAX motif) is

*This work was supported in part by National Institutes of Health Grants RO1 CA099506, RO1 AR059200, and HL41633 (to S. G. Y.) and GM46372 (to P. J. C.), and a grant from the University of California Tobacco-Related Disease Research Program (to M. O. B.). 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.

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1 The abbreviations used are: CAAX, motif where A is aliphatic amino acid; Rce1, Ras-converting enzyme 1; Icmt, isoprenylcysteine carboxyl methyltransferase; PMCA, polyinosinic-polycytidyllic acid: QPCR, quantitative polymerase chain reaction; ER, endoplasmic reticulum; H&E, hematoxylin/eosin; "lipidated" with a 15-carbon farnesyl or a 20-carbon geranyleranyl lipid. This processing step is carried out by cytosolic prenyltransferases (protein farnesyltransferase and protein geranyleranyltransferase type I). Second, a prenyl-protein-specific endoprotease of the endoplasmic reticulum, Ras-converting enzyme 1 (Rce1), clips off the last three amino acids from the protein (i.e. the –AAAX) (5–7). Third, the newly exposed C-terminal isoprenylcysteine is hydroxylated by isoprenylcysteine carboxyl methyltransferase (Icmt), a prenylprotein-specific, S-adenosylmethionine-dependent methytransferase of the endoplasmic reticulum (8–11).

Prenylation of CAAX proteins is vitally important to eukaryotic cells (1–4). Yeast lacking the shared o-chain of farnesyltransferase and geranyleranyltransferase type I are not viable (12). In mammalian cells, prenylation of CAAX proteins is absolutely required for their proper targeting to membrane surfaces and for proper protein function (1–4). The importance of protein farnesylation is clearly indicated by the fact that mice lacking farnesyltransferase die early during embryonic development (before embryonic day 6.5).2 The geranyleranyl-ylation of CAAX proteins is also critical for normal cellular function. Drugs that inhibit geranyleranyl-ylation of CAAX proteins have pronounced effects on cell growth and can trigger apoptosis (13–15). Further underscoring the importance of protein geranyleranyl-ylation, a cysteine protease from Yersinia pestis kills mammalian cells by clipping off the geranyleranyl-cysteine from specific CAAX proteins (16).

While the importance of prenylation for protein function and cell viability is well documented (1–4), the physiological importance of the second step of CAAX protein processing, the endoproteolytic trimming of the C terminus, has remained obscure. In yeast, Rce1 deficiency caused a partial mislocalization of Ras2p away from the plasma membrane, but there was no detectable effect on cell growth or vitality (5). In mammalian cells, Rce1 deficiency eliminated the endoproteolytic processing of the Ras proteins and led to a partial mislocalization of Ras proteins away from the plasma membrane (6, 17). However, Rce1 deficiency had only a marginal effect on fibroblast cell growth (17).

Studies of Rce1 knockout mice have heightened the mystery surrounding the importance of Rce1-mediated endoproteolytic processing for mammalian cells (6). These knockout mice grow and develop normally until late in embryonic development. After embryonic day 15.5, however, most of them die and those that are born alive are small and survive for only a few weeks.

2 M. Bergo and S. Young, unpublished observations.
Why Rce1 knockout mice die remains enigmatic, since no pathology was observed in any of the major organ systems (6). The fact that most do not survive postnatally indicates that CAAX protein endoproteolysis is somehow important; however, the survival of some Rce1-deficient mice for a few weeks after birth indicates that the endoproteolytic processing is not nearly as important as prenylation for the vitality of mammalian cells and tissues.

Hematopoietic stem cells harvested from the livers of adult mice, for example by examining tissue-specific knockout mice. Thus, the physiologic significance of Rce1 in adult animals is uncertain.

The finding that Rce1 deficiency leads to a partial mislocalization of Ras proteins within mouse fibroblasts (6) has encouraged efforts to develop Rce1 inhibitor drugs, with the hope that such drugs might diminish the effects of mutationally activated Ras proteins in human cancers (19, 20). A recent study indicated that Cre-mediated excision of Rce1 in mice limits Ras-induced transformation of cells (17), but much more research is required to determine if Rce1 would be a useful therapeutic target. In any case, if Rce1 inhibitor drugs were shown to be effective in blocking tumor growth in vivo, it would be essential to document that they could be given safely. In that regard, the question of whether the absence of CAAX protein endoproteolysis would adversely affect the function of vital organ systems in vivo has never been addressed.

In the current study, we sought to assess the physiologic importance of the endoproteolytic processing of prenylated CAAX proteins. To address this issue, we created mice lacking...
Rce1 in the heart, a tissue where Rce1 is expressed at very high levels (7). This study indicates that CAAX endoproteolysis is vitally important in the myocardium, as the absence of Rce1 in the heart caused a lethal-dilated cardiomyopathy.

EXPERIMENTAL PROCEDURES

Heart-specific Rce1 Knockout Mice—Mice with a “floxed” Rce1 allele (Rce1flx) were created in an earlier study to analyze the effect of an Rce1 gene excision on Ras-induced transformation of fibroblasts (17). In the Rce1flx allele, theloxP sites flank all of the protein-coding sequences of the gene; thus, Cre-mediated recombination eliminates the possibility of translation of a functional protein. To create an Rce1 knockout allele (Rce1flxΔ), Rce1flx mice were bred with transgenic mice that expressed Cre in the germline (21). As expected from earlier studies (6), mice with a single copy of the knockout allele (Rce1flxΔ) were phenotypically normal and free of pathology over 18 months of observation. Mice hemizygous for an α-myosin heavy chain-Cre transgene (αMyhc-Cre Δ) (22) were obtained from Dr. Michael Schneider (Baylor College of Medicine, Houston, TX).

To create heart-specific Rce1 knockout mice, we bred αMyhc-Cre Δ mice that contained both an Rce1 flx allele and an Rce1 Δ allele (Rce1flxΔ, αMyhc-Cre Δ). Two groups of mice were bred as controls: Rce1flx mice and αMyhc-Cre Δ mice that were homozygous for wild-type Rce1 alleles (Rce1 flx, αMyhc-Cre Δ). All mice were weaned at 28 days of age and maintained on a normal chow diet. The mice had a similar but mixed genetic background (>75% C57BL/6).

Assessing Cre-mediated Inactivation of Rce1 in the Heart—To assess the extent of Cre-mediated recombination within Rce1 in the heart-specific Rce1 knockout mice, BanHI-cleaved genomic DNA from the heart was analyzed with Southern blots (17). In addition, Rce1 expression in the heart was assessed by real-time quantitative PCR (QPCR).

For these studies, total RNA was isolated from the heart with a TRI REAGENT protocol (Molecular Research Center, Cincinnati, OH). Reverse transcription of RNA (1 μg) was performed with the SuperScriptII RNase H-Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA); the resulting cDNA (50 ng) was used for real-time QPCR reactions, performed for 40 cycles (95 °C for 15 s, 60 °C for 60 s) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The oligonucleotide primers were 5'-AAGGTGTCCTGCCCCTC-3' and 5'-TTTCGATGCCAGGCT-3', and the probe was 5'-6FAM-GTTCTTGGGCCCGCTGCCTCACAG-TAMRA-3' (PE Applied Biosystems). Rce1 expression levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (Rodent GAPDH Control Reagents, PE Applied Biosystems). Rce1 mRNA levels in the hearts of Rce1flxΔ,αMyhc-Cre Δ mice were compared with those in the hearts of control Rce1flxΔ mice.

Assessing Rce1 Activity in the Heart—CAAX endoprotease activity levels in heart tissue were measured with a coupled endoproteolytic processing...
methyl ester. Hearts of mice were excised, rinsed with cold phosphate-buffered saline, cut into multiple pieces, and then homogenized and sonicated in a buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 5 mM MgCl2, pH 7.4) containing protease inhibitors (Roche Applied Science, Nutley, NJ) (6, 7). After centrifugation at 1000 × g for 5 min and removal of tissue debris, heart extracts were collected. Extracts (300 μg) were incubated for 30 min at 37 °C in a total volume of 100 μl in the presence of human recombinant farnesyl-K-Ras (4 μM), the methyl donor S-adenosyl-L-[14C]methionine (1 mM, 80 Ci/mmol; Amersham Biosciences), and recombinant Icmt (8). The reaction was stopped by adding 100 μl of 1 N NaOH containing 1% SDS. A portion of the mixture (135 μl) was spotted onto a filter paper, which was wedged into the neck of a vial containing 5 ml of scintillation fluid. The vial was then capped and incubated overnight at room temperature. NaOH hydrolyzes the methyl esters, releasing [14C]methanol, which rapidly enters the scintillation fluid. After removal of the filter paper, the radioactivity in the scintillation fluid is measured. As a control, we tested the incorporation of S-adenosyl-L-[14C]methionine into nonfarnesylated K-Ras; the incorporation rates were invariably <5% of those into farnesyl-K-Ras.

Quantifying Substrate Accumulation in Rce1-deficient Fibroblasts and Heart Tissue—To assess the level of accumulation of Rce1 substrates in Rce1+/− fibroblasts, whole-cell lysates (50 μg) from Rce1−/− and Rce1+/− fibroblasts were mixed on ice and incubated with S-adenosyl-L-[14C]methionine at 37 °C for 45 min. In this experiment, Rce1 substrates within the Rce1−/− cells are cleaved by the Rce1 enzymatic activity in the Rce1+/− lysates (23). The endoproteolytic cleavage reaction renders the proteins susceptible to Icmt-mediated carboxyl methylation; the amount of methylation can be quantified by measuring the amount of [14C]methanol released after the addition of 100 μl of 1 N NaOH containing 1% SDS. As controls, we measured the methylation in lysates (100 μg) from either Rce1−/− or Rce1+/− fibroblasts. (In Rce1+/− lysates, the amounts of methylation would be expected to be low, since CAAX proteins would be fully processed (i.e. cleaved and methylated) prior to the incubation; in Rce1−/− lysates, the amount of methylation would be low because the absence of Icmt-mediated endoproteolysis precludes methylation of the isoprenylcysteine.)

To assess the level of Rce1 substrates in hearts, we incubated total heart lysates (500 μg) from Rce1flx/flx,Myhc-Cre−/− mice with S-adenosyl-L-[14C]methionine at 37 °C for 45 min. In this experiment, we relied on Rce1 in "non-cardiomyocytes" to cleave the Rce1 substrates in the Rce1-deficient cardiomyocytes, thereby rendering them susceptible to carboxyl methylation. The amount of methylation was quantified by measuring the base-releasable radioactivity. For controls, methylation was quantified in lysates from hearts of wild-type mice as well as mice deficient in Zmpste24, another integral membrane protease of the ER.

Activation of Erk1/2 in Rce1-deficient Cells—Serum-stimulated activation of Erk1/2 was assessed by seeding 1 × 105 Rce1−/− and Rce1+/− cells on 60-mm dishes followed by overnight serum starvation. The next morning, medium containing 10% serum was added to the cells. Cells were harvested at various time points after serum stimulation, and total cell lysates were analyzed by immunoblotting with an antibody recognizing phosphorylated Erk1/2 (phospho-p44/42 MAP kinase E10 monoclonal, Cell Signaling Technology, Beverly, MA), and total Erk1/2 (p44/42 MAP kinase, polyclonal, Cell Signaling Technology).

An identical series of experiments was performed with Rce1flx/flx fibroblasts and Rce1−/− fibroblasts (Rce1−/−) that were generated from them. To produce the Rce1flx/flx fibroblasts, Rce1flx/flx fibroblasts were treated with a Cre adenovirus (AdRSVCre) as described (17). In these experiments, the parental Rce1flx/flx fibroblasts were treated with a β-galactosidase adenovirus (AdRSVβGal).

Analyzing Heart-specific Rce1 Knockout Mice—Rce1flx/flx,Myhc-Cre−/− mice (n = 14), Rce1flx/flx mice (n = 10), and Rce1−/−,Myhc-Cre−/− mice (n = 10) were fed a normal diet for 10 months. Mice that died before 10 months of age were analyzed for gross and microscopic pathology. Hearts were excised, washed in phosphate-buffered saline, cut into multiple pieces, and then homogenized and sonicated in a buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 5 mM MgCl2, pH 7.4) containing protease inhibitors (Roche Applied Science, Nutley, NJ) (6, 7). After centrifugation at 1000 × g for 5 min and removal of tissue debris, heart extracts were collected. Extracts (300 μg) were incubated for 30 min at 37 °C in a total volume of 100 μl in the presence of human recombinant farnesyl-K-Ras (4 μM), the methyl donor S-adenosyl-L-[14C]methionine (1 mM, 80 Ci/mmol; Amersham Biosciences), and recombinant Icmt (8). The reaction was stopped by adding 100 μl of 1 N NaOH containing 1% SDS. As controls, we measured the methylation in lysates (100 μg) from either Rce1−/− or Rce1+/− fibroblasts. (In Rce1+/− lysates, the amounts of methylation would be expected to be low, since CAAX proteins would be fully processed (i.e. cleaved and methylated) prior to the incubation; in Rce1−/− lysates, the amount of methylation would be low because the absence of Icmt-mediated endoproteolysis precludes methylation of the isoprenylcysteine.)

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breeding Rce1flx/flx mice carrying the interferon-inducible Mx1-Cre transgene (26), and then inducing Cre expression with intraperitoneal injections of polyinosinic-polycytidylic ribonucleic acid (pI-pC, Sigma) (26–28).

Statistical Analysis—Data are expressed as means ± S.E., unless otherwise indicated. Differences between groups were analyzed with a two-tailed Student’s t test (Primer of Biostatistics, Version 3.0, McGraw Hill, 1992).

RESULTS

In earlier studies, we showed that Cre-mediated excision of Rce1 eliminates the enzymatic activity responsible for the endoproteolytic processing of Ras proteins, causing retarded mobility on SDS-polyacrylamide gels (6). However, an Rce1 gene excision had little effect on the growth of cultured fibroblasts (17). In the current project, we sought to define the consequences of inactivating Rce1 in the tissues of adult mice. We injected Cre adenovirus into the internal jugular vein of Rce1flx/flx mice, with the expectation that the adenovirus would be taken up almost exclusively by hepatocytes (after intravenous administration, adenovirus is taken up largely by the liver, Ref. 29). We further expected that Cre in the liver would excise the coding sequences of Rce1 and reduce CAAX endoprotease activity. Indeed, Cre adenovirus did cause a significant decrease in CAAX endoprotease expression in the liver (Fig. 1A). Western blot analysis of the Ras proteins in the liver revealed that approximately one-half of the Ras proteins had retarded electrophoretic mobility (Fig. 1B), indicating the complete loss of endoproteolytic processing in a significant fraction of the liver cells. We also inactivated Rce1 in the liver by breeding Rce1flx/flx, Mx1-Cre/+ mice and then inducing Cre expression with injections of pl-pC (26–28). As judged by Southern blots, the excision of Rce1 in the livers of the pl-pC-treated mice was complete (Fig. 1C), and Rce1 activity levels were dramatically reduced (Fig. 1D). Despite the absence of Rce1 in the liver DNA, the pl-pC-treated Rce1flx/flxMx1-Cre/+ mice gained weight and exhibited normal vitality over 2–3 months of observation. During this time, transaminase levels remained normal, and the histological appearance of the liver on hematoxylin/eosin-stained sections was indistinguishable from that of wild-type mice (not shown).

To further explore the physiologic importance of CAAX protein endoproteolysis, we created mice lacking Rce1 in the heart, where Rce1 is expressed at very high levels (7). To generate the heart-specific Rce1 knockout mice, we bred Rce1flx/flx mice harboring a Cre transgene under the control of the αMyhc promoter (22, 30). As expected, Southern blots of genomic DNA from the hearts of Rce1flx/flxαMyhc-Cre/+ mice revealed incomplete recombination (Fig. 2A), which was not surprising, since the majority of cells within the heart are not myocytes (31, 32). We suspected that our best chance to achieve a near-complete inactivation of Rce1 in cardiac myocytes would be to breed αMyhc-Cre+/− mice harboring one Rce1 knockout allele (Rce1flx) and one “floxed” allele (Rce1flx/αMyhc-Cre+/−). In those myocytes, Cre-mediated inactivation of a single Rce1flx allele would eliminate all Rce1 expression. The presence of the Rce1flx allele in the genomic DNA obviously made it more difficult to discern Cre-mediated inactivation of the Rce1flx allele with Southern blots (Fig. 2A). However, the inactivation of Rce1 in the heart could be documented easily with QPCR studies and by measuring CAAX endoprotease activity levels. QPCR revealed significantly fewer Rce1 transcripts in Rce1flx/αMyhc-Cre+/− hearts than in Rce1flx/αMyhc control hearts (Fig. 2B). Consistent with these data, we also found a significant reduction in CAAX endoprotease activity levels in heart extracts from Rce1flx/αMyhc-Cre−/+ mice (Fig. 2C). The reduced Rce1 mRNA levels and CAAX endoprotease activity levels demonstrate that the αMyhc-Cre transgene was effective in inactivating Rce1 in the heart. The less-than-complete elimination of Rce1 transcripts and endoprotease activity was not surprising in view of the fact that the αMyhc-Cre transgene is expressed only in the myocytes of the heart and not in other cell types (e.g. endothelial cells and fibroblasts). As expected, we observed a double Ras band in the heart tissue from Rce1flx/αMyhc-Cre−/+ mice (Fig. 2D), suggesting a complete loss of endoproteolytic processing in a significant fraction of cardiac myocytes.

The Rce1flx/αMyhc-Cre−/+ mice appeared healthy at weaning, but started dying by 3–5 months of age (Fig. 3). By 7 months of age, 50% of the Rce1flx/αMyhc-Cre−/+ mice had died; by 10 months, 70% had died. In contrast, none of the Rce1flx mice or Rce1flx/αMyhc-Cre−/+ mice died during a 10-month...
follow-up period (Fig. 3). Similarly, we never observed any premature deaths in our colony of Rce1\textsuperscript{-}\/omice.

Several weeks before their deaths, most of the heart-specific Rce1 knockout mice were listless and had ruffled fur. Although we did not observe edematous extremities in the mice, we always noted a sizable accumulation of pleural and peritoneal fluid at autopsy, and the hearts were invariably enlarged (Fig. 4A). Echocardiography showed dilated left ventricles (not shown). Histological sections revealed dilatation of all four chambers of the heart, and organized thrombi were occasionally noted within the left atrium (Fig. 4B). The left ventricular musculature was thin and dystrophic, and there were increased amounts of collagen between ventricular myocytes (Fig. 5). Heart tissue from Rce1\textsuperscript{flx/flx}, Rce1\textsuperscript{-}\/+\alphaMyhc-Cre\textsuperscript{+/o}, and Rce1\textsuperscript{flx/flx} control mice was histologically normal.

The striking cardiomyopathy in the Rce1\textsuperscript{flx/flx}\alphaMyhc-Cre\textsuperscript{+/o} mice (along with the normal heart histology and normal survival in the control groups) suggested that the absence of Rce1-mediated endoproteolytic processing was mechanistically related to the development of cardiomyopathy. However, such a mechanism would clearly be open to criticism without firm biochemical evidence that Rce1 substrates actually accumulate in the hearts of Rce1\textsuperscript{flx/flx}\alphaMyhc-Cre\textsuperscript{+/o} mice. We therefore developed a coupled endoproteolysis/methylation assay to document the accumulation of Rce1 substrates in Rce1-deficient cells and tissues. This assay measures the ability of Rce1 to cleave accumulated Rce1 substrates, rendering them susceptible to Icmt-mediated methylation.

To show that this strategy was feasible, we first asked if Rce1 substrates accumulate in Rce1-deficient fibroblasts. Indeed, they did (Fig. 6A). When whole-cell lysates from Rce1\textsuperscript{-}\/+ and Rce1\textsuperscript{-}/- fibroblasts were incubated in the presence of S-adenosyl-L-[methyl-\textsuperscript{14}C]methionine, the accumulated Rce1 substrates in the Rce1\textsuperscript{-}/- cells were cleaved, allowing them to be methylated (Fig. 6A). The methylation could be readily quantified with a base-hydrolysis assay. In contrast, incubating Rce1\textsuperscript{-}/- lysates with S-adenosyl-L-[methyl-\textsuperscript{14}C]methionine yielded negligible levels of methylation, reflecting the fact that most CAAX proteins in those cells had already been cleaved and methylated (Fig. 6A). Similarly, incubating Rce1\textsuperscript{-}/- lysates in the presence of S-adenosyl-L-[methyl-\textsuperscript{14}C]methionine yielded very low levels of methylation because the absence of Rce1 prevents methylation (Fig. 6A).

Next, we tested whether Rce1 substrates accumulate in the hearts of Rce1\textsuperscript{flx/flx}\alphaMyhc-Cre\textsuperscript{+/o} mice. For these studies, we relied on the fact that many cells in Rce1\textsuperscript{flx/flx}\alphaMyhc-Cre\textsuperscript{+/o} hearts (e.g. endothelial cells and fibroblasts) express Rce1 at wild-type levels. Thus, when fresh Rce1\textsuperscript{flx/flx}\alphaMyhc-Cre\textsuperscript{+/o} lysates were incubated with S-adenosyl-L-[methyl-\textsuperscript{14}C]methionine, we predicted that the Rce1 from the endothelial cells and fibroblasts would cleave the accumulated substrates in the Rce1-deficient cardiomyocytes, allowing methylation to occur. Indeed, this prediction was upheld. We observed far more methylation in the lysates from Rce1\textsuperscript{flx/flx}\alphaMyhc-Cre\textsuperscript{+/o} mice than in lysates from wild-type controls and from mice lacking another ER endoprotease, Zmpste24 (Fig. 6B). These data clearly show that Rce1 substrates accumulate in hearts of Rce1\textsuperscript{flx/flx}\alphaMyhc-Cre\textsuperscript{+/o} mice.

We hypothesized that absent endoproteolysis of the Ras proteins might result in impaired growth factor-mediated activation of the Ras effector Erk1/2, contributing to the development of cardiomyopathy. However, growth factor-mediated activation of Erk1/2 was no different in Rce1\textsuperscript{-}/- and Rce1\textsuperscript{-}/- cells (Fig. 7A). We were concerned that intrinsic genetic differences between independent lines of Rce1\textsuperscript{-}/- and Rce1\textsuperscript{-}/- fibroblasts might conceivably have prevented us from observing subtle differences in Erk1/2 activation. Accordingly, we repeated the Erk1/2 activation experiments in parental Rce1\textsuperscript{flx/flx} cells and Rce1\textsuperscript{-}/- cells that were derived from them (i.e. two cell lines that were genetically identical except for the difference in Rce1 expression). Once again, however, we observed no differences in Rce1-expressing and Rce1-deficient cells in Erk1/2 activation in response to serum or epidermal growth factor (Fig. 7B).

**DISCUSSION**

While the physiologic importance of protein prenylation for cell homeostasis has become axiomatic (1, 2), the physiologic relevance of the subsequent step in CAAX protein processing,
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endoproteolysis, has remained mysterious (4). In the current study, we demonstrate that the endoproteolytic processing of CAAX proteins is critically important in the heart. Mice lacking Rce1 expression in cardiac myocytes develop a dilated cardiomyopathy, beginning as early as 3 months of age. By 10 months of age, most of the heart-specific Rce1 knockout mice had died. Pathologic studies uncovered findings that are common to lethally irradiated recipient mice and manifested normal expression in cardiac myocytes develop a dilated cardiomyopathy (33). The clear-cut importance of thrombi, and an increased amount of fibrous tissue between

Fig. 7. Normal serum-stimulated activation of Erk1/2 in Rce1−/− (A) and Rce1flx/flx (B) fibroblasts. Fibroblasts were serum-starved overnight. The next morning, serum-containing medium was added to the cells (time 0). At various time points, cells were harvested and analyzed by immunoblotting with an antibody against phosphorylated Erk1/2. The blots were stripped and incubated with an antibody recognizing total Erk1/2 as a loading control. The experiment was repeated three times with similar results. Also, similar results were obtained when Erk1/2 was stimulated with epidermal growth factor rather than serum.

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J. Biol. Chem. 2004, 279:4729-4736.
doi: 10.1074/jbc.M310081200 originally published online November 18, 2003

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