Genetic analyses of Rce1 function in Ras membrane association and function

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Abstract

Proteins terminating with a *CAAX* motif, such as the nuclear lamins and the Ras family of proteins, undergo posttranslational modification of a carboxyl-terminal cysteine with an isoprenyl lipid—a process called protein prenylation. After prenylation, the last three residues of *CAAX* proteins are clipped off by an endoprotease of the endoplasmic reticulum. Rce1 is responsible for the endoproteolytic processing of the Ras proteins and is likely responsible for endoproteolytic processing of the vast majority of *CAAX* proteins. Prenylation has been shown to be essential for the proper intracellular targeting and function of several *CAAX* proteins, but the physiologic importance of the endoprotease step has remained less certain. Here, we will review methods that have been used to define the physiologic importance of the endoproteolytic processing step of *CAAX* protein processing.

Introduction

Eukaryotic proteins that terminate with a *"CAAX"* sequence typically undergo a series of posttranslational processing reactions, beginning with protein prenylation, which is the attachment of a farnesyl or geranylgeranyl lipid to the thiol group of the cysteine residue (the *"C"* of the *CAAX* motif) (Zhang and Casey, 1996). Second, the last three amino acids of the protein (*i.e.,* the *–AAX*) are removed by a prenylprotein-specific endoprotease of the endoplasmic reticulum (ER) (Ashby, 1998; Boyartchuk et al., 1997; Young et al., 2000). Third, the carboxyl group of the newly exposed isoprenylcysteine is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT) (Dai et al., 1998; Hrycyna et al., 1991; Young et al., 2000). These three processing steps have been studied intensively with yeast **a**-factor, a farnesylated yeast mating pheromone, and with the yeast and mammalian Ras proteins, small GTP-binding proteins involved in signal transduction. However, large numbers of cellular proteins with diverse biological functions contain a *CAAX* motif and undergo these modifications.

The enzymes responsible for the prenylation of the *CAAX* proteins, protein farnesyltransferase and protein geranylgeranyltransferase I, have been characterized extensively in both yeast and higher organisms (Zhang and Casey, 1996). Progress in understanding the importance of the endoprotease step has lagged behind, but during the past few years some progress has been made in understanding the *CAAX* endoproteases and their functional importance.

Two genes from *Saccharomyces cerevisia*e, *RCE1* and *STE24 (AFC1)*, were identified as playing a role in the endoproteolytic removal of the "-*AAX*" from Ras2p and the precursor to the yeast mating pheromone **a**-factor (Boyartchuk et al., 1997). Rce1p (for Ras and **a**-factor converting enzyme) is essential for cleaving the –*AAX* from Ras2p. In addition, Rce1p is quite capable of cleaving the –*AAX* from **a**-factor. Ste24p is a zinc metalloproteinase that plays dual roles in the endoproteolytic processing of the precursor to **a**-factor. Along with Rce1p, Ste24p is capable of carrying out the carboxyl-terminal cleavage reaction (*i.e.,* the release of the – *AAX* from **a**-factor) (Boyartchuk et al., 1997; Tam et al., 1998). In addition, however, it cleaves seven amino acids from the amino terminus of the protein (Tam et al., 1998). Ste24p plays absolutely no role in the endoproteolytic processing of the yeast Ras proteins (Boyartchuk et al., 1997). In fact, **a**-factor is the only well-established substrate for Ste24p in yeast (Boyartchuk et al., 1997).

Both yeast Rce1p and Ste24p are endoplasmic reticulum proteins with multiple transmembrane domains (Young et al., 2000). Rce1p is 315-amino acids in length and is predicted to contain multiple transmembrane helices (Boyartchuk et al., 1997). The Rce1p amino acid sequence does not contain sequence motifs characteristic of the well-defined classes of proteases, but there are remote similarities with the type IIb signal peptidase, which cleaves signal sequences from proteins containing nearby lipid modifications (Boyartchuk et al., 1997). Ste24p contains 453 amino acids and also contains multiple transmembrane domains (Boyartchuk et al., 1997). Ste24p contains a HEXXH (H, His; E, Glu) motif between amino acids 297–301, a sequence shared by many zinc-dependent metalloproteases. Mutating either of the conserved histidines in the HEXXH motif within Ste24p blocks **a**-factor processing (Boyartchuk et al., 1997; Fujimura-Kamada et al., 1997)

In yeast, Rce1p is essential for Ras processing and function (Boyartchuk et al., 1997). Heat shock sensitivity elicited by a mutationally activated Ras2p is suppressed by *RCE1* deficiency. However, *RCE1* deficiency in yeast almost certainly does not completely block all Ras functions, given that *rce1Δ* yeast are quite viable (Boyartchuk et al., 1997) and yeast lacking the Ras proteins are not (Kataoka et al., 1984). *RCE1* deficiency in yeast mislocalizes Ras2p away from the plasma membrane to the cytosol (Boyartchuk et al., 1997). In wild-type yeast, a green fluorescent protein (GFP)–*RAS2* fusion is targeted to the periphery of the cells (*i.e.*, the plasma membrane). In contrast, the GFP–*RAS2* fusion in *rce1*α yeast is located inside the cell (*i.e.*, either in the cytosol or associated with internal membrane compartments) (Boyartchuk et al., 1997).

The identification of *RCE1* and *STE24* in yeast made it possible to identify the human and mouse orthologues, *RCE1* and *ZMPSTE24* (Boyartchuk et al., 1997; Fujimura-Kamada et al., 1997; Kumagai et al., 1999; Leung et al., 2001; Young et al., 2000). Human RCE1 is absolutely required for normal processing of the Ras proteins; the *RCE1* gene and is expressed ubiquitously, with the highest levels of expression in placenta, heart, and skeletal muscle (Kim et al., 1999; Otto et al., 1999). Human RCE1 is 329 amino acids in length, 27.6% identical to Rce1p in *S. cerevisiae,* and contains multiple transmembrane helices (Young et al., 2000). The human orthologue for yeast *STE24*, *ZMPSTE24*, is also expressed ubiquitously (Bergo et al., 2002b; Leung et al., 2001). It is 475 amino acids in length, with 36% identity to the yeast protein (Young et al., 2000). Like yeast Ste24p, human ZMPSTE24 contains the zinc metalloprotease motif HEXXH and multiple transmembrane helices (Young et al., 2000). Human and mouse ZMPSTE24 faithfully carries out the processing of yeast **a**-factor (Leung et al., 2001); however, no **a**-factor orthologues have been identified in mammals. During the past few years, several laboratories have shown that Zmpste24 is critical for the endoproteolytic processing of prelamin A, a farnesylated *CAAX* protein that is the precursor to lamin A, a structural protein of the nuclear envelope (Bergo et al., 2002b; Pendás et al., 2002). Akin to Ste24p-processing of **a**-factor, Zmpste24 is almost certainly capable of cleaving the –*AAX* from

prelamin A (Bergo et al., 2002b; Corrigan et al., 2004). In addition, it cleaves prelamin A a second time, 15 residues upstream from the farnesylcysteine, releasing mature lamin A (Corrigan et al., 2004; Dalton and Sinensky, 1995; Kilic et al., 1997). Remarkably, prelamin A and yeast **a**-factor are apparently cleaved twice by Zmpste24 in a highly specific fashion, despite very little amino acid similarity surrounding the four different sessile bonds.

Establishing that Human RCE1 is a *CAAX* Endoprotease

The laboratory of Patrick Casey (Duke University) expressed human RCE1 protein *Sf9* insect cells and performed biochemical assays to show that it was a *CAAX* endoprotease (Kim et al., 1999; Otto et al., 1999). Extracts from the *Sf9* cells were mixed with recombinant isoprenylated *CAAX* proteins. In this assay, recombinant RCE1 cleaves the –*AAX* from the isoprenylated proteins, rendering the proteins susceptible to methylation with recombinant Ste14p (the yeast isoprenylcysteine carboxyl methyltransferase) and [*methyl*-^{3}H]-*S*-adenosylmethionine. With this "coupled endoproteolysis/methylation assay," Casey's laboratory demonstrated that human RCE1 is capable of processing farnesylated K-Ras, farnesylated H-Ras, farnesylated N-Ras, the farnesylated heterotrimeric G protein $G_{\alpha 1}$ subunit, geranylgeranylated K-Ras, and geranylgeranyl-Rap1B. Both farnesylated and geranylgeranylated K-Ras exhibited a K_m value of approximately 0.5 μM and similar K_{cat} values. Prenylated *CAAX* peptides, but not nonisoprenylated peptides, were able to compete for the processing of isoprenylated K-Ras (Otto et al., 1999). Also, a previously identified inhibitor of endoproteolytic processing, RPI, was shown to be an effective inhibitor of RCE1, with an IC_{50} of ~5 nM (Otto et al., 1999).

Rce1 Knockout Mice

The laboratory of Stephen Young (Univ. California, Los Angeles) created *Rce1*-deficient mice with standard gene-targeting techniques (Kim et al., 1999). Heterozygous knockout mice ($Rce1^{+/-}$) were normal. However, almost all of the homozygous embryos ($Rce1^{-/-}$) died, beginning at about embryonic day 15 (E15). $Rce1^{-/-}$ mice were born alive very rarely, and those mice were invariably quite small and died within a few weeks. The precise reason for the death of $Rce1^{-/-}$ mice is not known; the viable $Rce1^{-/-}$ embryos at E15.5 revealed no consistent differences in morphology, organogenesis, stage of development, color, or size.

Analyzing Ras Function in *Rce1*$^{-/-}$ MEFs

Primary mouse embryonic fibroblasts (MEFS) could be cultured from the $Rce1^{-/-}$ embryos (Kim et al., 1999). To generate fibroblasts, $Rce1^{+/-}$ mice (which can be obtained from the authors of this chapter), are intercrossed, and pregnant females are sacrificed at E13.5. Genotyping of embryos is performed by Southern blot analysis of DNA prepared from fetal membranes (Kim et al., 1999). The embryos are incubated in 5.0 ml of 0.25% trypsin-EDTA (Life Technologies, Gaithersburg, MD) for 8 h at 4°C, followed by a 20-min incubation at 37° C. The embryos are mechanically disrupted by repeated pipetting in 5 ml of DME-FBS medium [Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal bovine serum (FBS), L-glutamine, nonessential amino acids, penicillin-streptomycin (all from Life Technologies), and 2-mercaptoethanol (Sigma, 100 μM final concentration)]. The debris is allowed to settle, and the cell suspension, diluted to 25 ml, is plated in T150 tissue culture flasks. The cells are grown in an incubator at 37°C with 7% CO_2. (~1 α 10^7 cells are obtained from each embryo) To immortalize the MEFS, cells are passaged according to a 3T6 protocol (Todaro, 1969), in which cells are harvested and counted every 3 days and replated at a density of 2 α 10^6 cells per 100-mm dish.

Absence of *CAAX* Endoprotease Activity in *Rce1*-deficient Cells

CAAX endoprotease activity in $Rce1^{-/-}$ and $Rce1^{+/+}$ MEFs can be measured with a coupled endoprotease/methylation assay (Kim et al., 1999; Otto et al., 1999). Substrate proteins (*e.g.,* K-Ras, N-Ras and Rap-1B) containing a *CAAX* sequence are expressed in *Escherichia coli* and then prenylated *in vitro* with either recombinant protein farnesyltransferase or protein geranylgeranyltransferase (Otto et al., 1999). To prepare membranes from $Rce1^{+/+}$ and $Rce1^{-/-}$ embryonic fibroblasts, cells were lysed by sonication. After centrifugation at 500 α *g* for 10 min (to remove debris and cell nuclei), the membrane fraction is prepared by spinning at 200,000 α *g* for 1.5 h. The membranes are then resuspended in 50 mM Tris-HCl at protein concentrations of 10–25 mg/ml. To measure the prenylprotein–specific *CAAX* endoprotease activity, the prenylated protein substrates and membrane fractions are mixed in 50 μl of a 100 mM Hepes buffer (pH 7.5) containing 5 mM $MgCl_2$. After a 30-min incubation at 37°C, the proteolysis reactions are stopped and the methyltransferase reaction is initiated by adding a cocktail (20 μl) containing 5 mM $NaHPO_4$, 87.5 mM EDTA, several protease inhibitors (300 μM N-tosyl-ʟ-phenylalanine chloromethylketone, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM 1,10-phenanthroline), 20 μg of *Sf9* cell membranes expressing yeast *STE14*, and 17.5 μM [^{3}H] AdoMet (1.5 Ci/mmol). After a 20-min incubation at 37°C, the methylation reaction is terminated by adding 0.5 ml of 4% sodium dodecyl sulfate (SDS). Bovine brain cytosol (50 μg protein) is added as a carrier, and the mixture is incubated for 20 min at room temperature, after which the proteins were precipitated with 0.5 ml of 30% trichloroacetic acid. Precipitated proteins are collected on glass fiber or nitrocellulose filters and the extent of proteolysis is determined by quantifying ^{3}H incorporation into the protein by scintillation counting. With this assay, $Rce1^{-/-}$ membranes were shown to lack *CAAX* processing activity for farnesylated heterotrimeric $G_{\alpha 1}$ subunit, farnesylated H-Ras, farnesylated N-Ras, geranylgeranylated K-Ras, and geranylgeranyl-Rap1B (Kim et al., 1999; Otto et al., 1999).

Documenting an Accumulation of Uncleaved Rce1 Substrates in *Rce1*-deficient Cells

$Rce1^{-/-}$ MEFs accumulate uncleaved Rce1 substrates, and this accumulation of Rce1 substrates can be detected with a coupled endoproteolysis/methylation assay (Leung et al., 2001). Whole-cell extracts (150 μg) from $Rce1^{+/+}$ and $Rce1^{-/-}$ MEFs are incubated with 10 μM *S*-adenosyl-ʟ-[*methyl*-^{14}C] methionine (55 Ci/mol, Amersham Pharmacia), *Sf9* cells expressing high levels of Rce1 (100 μg), and *Sf9* cells expressing high levels of yeast Ste14p (100 μg). The reaction is incubated for 2 h at 37°C. The reaction is stopped by adding 50 μl of 1.0 M NaOH containing 0.1% SDS. The reaction mixture (90 μl) can then be spotted onto a pleated 2 α 8-cm filter paper wedged in the neck of a 20-ml scintillation vial containing 5 ml of scintillation fluid (ScintiSafe Econo 1, Fisher). The vials are capped and incubated at room temperature for 5 h to allow the [^{14}C] methanol (formed by base-hydrolysis of methyl esters) to diffuse into the scintillation fluid. The amount of base-releasable methanol is far higher in the $Rce1^{-/-}$ fibroblasts then in the $Rce1^{+/+}$, reflecting the accumulation of uncleaved Rce1 substrates (substrates that can be readily cleaved and methylated in the setting of high levels of Rce1 and Ste14p) (Fig. 1). Importantly, there was no accumulation of "methylatable" protein substrates in fibroblasts lacking *Zmpste24* (Leung et al., 2001) (Fig. 1).

Altered Electrophoretic Migration of Ras proteins in *Rce1*⁻/⁻ Fibroblasts

The electrophoretic migration of the Ras proteins in $Rce1^{-/-}$ cells is abnormally slow, reflecting an absence of endoproteolytic processing (Kim et al., 1999). To study the electrophoretic migration of Ras proteins, it is first necessary to prepare lysates of MEFs (Kim et al., 1999). Confluent cultures of MEFS are harvested from a 100-mm plate in 0.5 ml of RIPA lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium

deoxycholate, 0.1% SDS, 0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml of aprotinin] and disrupted by sonication for 10 s in a Branson model 400 sonifier (duty cycle constant, output control 0.3; Danbury, CT). The samples are then heated to 95°C for 5 min and loaded onto an SDS/10–20% gradient polyacrylamide gel. After electrophoretic transfer of the proteins to a nitrocellulose membrane, western blotting is performed with the "pan-Ras" monoclonal antibody Ab-4 (*i.e.,* binds to N-, K-, and H-Ras; Oncogene Science, Uniondale, NY) or the K-Ras-specific monoclonal antibody Ab-1 (Oncogene Science), followed by an incubation with horseradish peroxidase–conjugated sheep anti-mouse IgG (Amersham). The binding of the secondary antibodies is detected by enhanced chemiluminescence (Amersham). The electrophoretic mobility of the Ras proteins from *Rce1*$^{-/-}$ MEFs (or *Rce1*$^{-/-}$ embryos) is distinctly abnormal, even on a minigel. In fact, Ras proteins with a normal electrophoretic mobility are undetectable in *Rce1*$^{-/-}$ cells (Fig. 2). When equal amounts of *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ MEF lysates are mixed, one observes a doublet Ras band, consisting of the processed Ras proteins in *Rce1*$^{+/+}$ MEFs and the unprocessed Ras proteins in *Rce1*$^{-/-}$ MEFs (Kim et al., 1999).

Methylation of Ras Proteins in *Rce1*$^{-/-}$ Fibroblasts

The absence of Ras endoproteolysis means that the Ras proteins cannot be methylated. The absence of a methyl ester in the Ras proteins from *Rce1*$^{-/-}$ MEFs can be documented by a metabolic labeling assay (Clarke et al., 1988) (Kim et al., 1999). 100-mm dishes containing approximately $2.5 \alpha 10^6$ *Rce1*$^{-/-}$ or *Rce1*$^{+/+}$ MEFs are incubated in the presence of 1 mCi of L-[*methyl*-^{3}H]methionine (80Ci/mmol, Amersham-Pharmacia, Chicago, IL) and [^{35}S] cysteine (>1000 Ci/mmol, Amersham-Pharmacia) in methionine- and cysteine-free DMEM/10% FBS in a 7% CO_2 incubator at 37°C for 24 h. The medium is removed, and the cells are washed with 5 ml of ice-cold PBS, scraped from the dish into 1 ml of ice-cold PBS, and centrifuged at $500 \alpha g$. Cell pellets are resuspended in 1.0 ml of RIPA lysis buffer and disrupted by sonication, as described above. Ras proteins are then immunoprecipitated with antibody Y13-259, and the immunoprecipitate is size-fractionated on an SDS/10–20% polyacrylamide gel. The gel is fixed in isopropanol, water, and acetic acid (25:65:10, v/v), soaked for 30 min in Amplify (Amersham-Pharmacia), and dried. The gel is imaged with a phosphorimager, and the region of the gel corresponding to the Ras band is excised. The amount of [^{3}H] methanol released from the gel fragment by base hydrolysis of the farnesylcysteine methyl esters is quantified by scintillation counting. The gel slice is placed in a 1.5-ml capless microcentrifuge tube and mixed with 200 μl of 2 M NaOH; the tube is lowered into 20-ml scintillation vial containing 6 ml of Safety-Solve II counting fluor (RPI) fluid. The scintillation vial is then capped and incubated at 55°C. The [^{3}H]methanol released from the farnesylcysteine methyl esters diffuses into the fluor within the capped vial, while the ^{35}S-labeled protein remains in the gel slice. After 24 h, the microcentrifuge tubes are removed, and the radioactivity in the fluor is counted. As judged by the [^{3}H]methanol release, the Ras proteins from *Rce1*$^{+/+}$ cells contain a ^{3}H-labeled farnesylcysteine methyl ester, whereas Ras proteins from the *Rce1*$^{-/-}$ cells do not (Kim et al., 1999).

Subcellular Fractionation and Localization of Ras Proteins in *Rce1*$^{-/-}$ MEFs

To determine the degree of membrane association of Ras proteins in *Rce1*$^{-/-}$ MEFs, subcellular fractionation studies can be performed (Bergo et al., 2002b; Bergo et al., 2000; Kim et al., 1999). 100-mm dishes containing confluent MEFs are washed with ice-cold PBS, and cells are collected in 1.0 ml of PBS and centrifuged at $500 \alpha g$ for 10 min. Cell pellets are incubated with 1225 μl of hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1.0 mM $MgCl_2$, 0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml of aprotinin, and 1μM DDT) and placed on ice for 10 min. Cells are disrupted with an ice-cold dounce tissue homogenizer, after which 225 μl of 1 M NaCl is added. A total of 450 μl of this solution ("total lysate") is transferred to a microfuge

tube and set aside. The remaining 1000 µl is transferred to a polycarbonate ultracentrifuge tube and spun at 100,000 α *g* for 30 min at 4°C. The supernatant fluid (S100, representing the cytosolic fraction) is transferred to a new microcentrifuge tube; the pellet (P100, representing the membrane fraction) is resuspended in 850 µl of hypotonic buffer and 150 µl of 1 M NaCl. Next, 50 µl of 10αRIPA buffer is added to the "total lysate" sample, and 110 µl is added to the S100 and P100 fractions. After incubation on ice for 10 min, the lysates are clarified by centrifugation at 25,000 α g for 30 min at 4°C. Supernatants are transferred to new microfuge tubes, and immunoprecipitation, gel fractionation, and western blot detection of the Ras proteins are performed as described earlier. A substantial difference in the intracellular distribution of the Ras proteins is apparent by immunoblot analysis of P100 and S100 fractions of *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ fibroblasts (Kim et al., 1999). Virtually all of the Ras proteins in *Rce1*$^{+/+}$ cells are located in the P100 fraction, whereas about one-half of the Ras proteins in *Rce1*$^{-/-}$ cells is located in the S100 fraction (Fig. 2).

To further assess the association of the Ras proteins with membranes of *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ embryonic fibroblasts, enhanced GFP–Ras fusion protein constructs can be expressed in MEFs and then visualized by fluorescence microscopy (Bergo et al., 2002a; Kim et al., 1999). *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ MEFs are transfected with 2.0 µg of GFP–Ras fusion plasmids with SuperFect Reagent (Qiagen, Valencia, CA). Cells are fixed in 4% formalin 24–28 h after transfection, mounted, and viewed by confocal microscopy. In *Rce1*$^{+/+}$ cells, the fluorescence is localized almost entirely to the plasma membrane. In contrast, the vast majority of the fluorescence in the *Rce1*$^{-/-}$ cells is cytosolic or associated with internal membranes. However, very small amounts of fluorescence can be observed along the plasma membrane in the *Rce1*$^{-/-}$ cells.

The importance of Rce1 for the localization of GFP-Ras fusion proteins is dependent on the fact that the Ras proteins are farnesylated. When mutations are introduced into the *CAAX* motif so as to create GFP-Ras fusions that are geranylgeranylated, the impact of *Rce1* deficiency on intracellular localization is no longer detectable (Michaelson et al., 2005). Thus, geranylgeranylated GFP–N-Ras and geranylgeranylated GFP–K-Ras are largely localized to the plasma membrane in *Rce1*$^{-/-}$ MEFs and not mislocalized to the cytosol (*i.e.*, a pattern indistinguishable from that in *Rce1*$^{+/+}$ cells). Changing the isoprenyl chain length of Rac1 and RhoB (proteins that are normally geranylgeranylated) had the opposite effect. The intracellular localization of geranylgeranylated GFP–Rac1 and geranylgeranylated GFP–RhoB is unaffected by *Rce1* deficiency. However, the intracellular localization of farnesylated GFP–Rac1 and farnesylated GFP–RhoB is sensitive to *Rce1* deficiency (Michaelson et al., 2005).

Defining the Impact of *Rce1* Deficiency on MAP Kinase Activation in *Rce1*$^{-/-}$ Fibroblasts

We suspected that absent endoproteolysis of the Ras proteins might result in impaired growth factor–mediated activation of the Ras effector Erk1/2. Serum-stimulated activation of Erk1/2 was assessed by seeding 1 α 10^5 *Rce1*$^{+/+}$ and *Rce1*$^{-/-}$ cells on 60-mm dishes followed by overnight serum starvation (Bergo et al., 2004). 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). Contrary to our expectation, we observed no effect of *Rce1* deficiency on the activation of Erk1/2 (*i.e.*, activation was no different in *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ cells). We were concerned that intrinsic genetic differences between independent lines of immortalized *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ fibroblasts might conceivably have prevented us from observing subtle differences in Erk1/2 activation. Accordingly, we repeated the Erk1/2 activation experiments

in *Rce1*^{flx/flx} cells (which have normal levels of *Rce1* expression) and the *Rce1*-deficient *Rce1*^{α/α} cells that were derived from them by Cre expression (*i.e.* two fibroblast cell lines that were identical except for *Rce1* expression). Once again, however, we observed no differences in *Rce1*-expressing and *Rce1*-deficient cells in Erk1/2 activation, either in response to serum or epidermal growth factor (Bergo et al., 2004).

Impact of Endoproteolysis on the Binding of K-Ras to Microtubules

The laboratory of Pat Casey identified a specific and prenylation-dependent interaction between tubulin/microtubules and K-Ras, but not H-Ras or several other small GTPases (Thissen et al., 1997). In a follow-up study, they found that the interaction between K-Ras and microtubules is highly dependent both on the polylysine domain with K-Ras as well as endoproteolysis and methylation at the carboxyl terminus (Chen et al., 2000). Partially processed K-Ras that was farnesylated (but retained the –AAX residues) bound microtubules. However, surprisingly, endoproteolytic removal of the –AAX from K-Ras abolished all binding. Even more surprising, the binding of K-Ras to microtubules was restored by methylation of the C-terminal prenylcysteine. Consistent with these results, localization of the green fluorescent protein–K-Ras fusion was paclitaxel-sensitive in cells lacking *Rce1*, while no paclitaxel effect was observed in cells lacking the methyltransferase Icmt. These studies show that the polylysine domain is critical for the interaction between K-Ras and tubulin/microtubules and provide the first evidence for a functional consequence of CAAX-processing steps (Chen et al., 2000).

Defining the Impact of *Rce1* Deficiency on Cell Growth and on Transformation by Activated Forms of Ras

To study the role of *Rce1* on cell growth and Ras-transformation, we examined *Rce1*^{+/+} and *Rce1*^{−/−} MEFs. We also constructed a conditional knockout allele in which the entire *Rce1* coding sequence was "floxed" (flanked by *loxP* sites). Mice homozygous for the conditional allele (*Rce1*^{flx/flx}) were viable, healthy, and fertile (Bergo et al., 2002a). *Rce1* ^{flx/flx} MEFs were produced, and it was possible to completely delete the *Rce1* gene from these cells by treating the cells with *Cre* adenovirus (thereby creating *Rce1*^{α/α} MEFs). Treating *Rce1* ^{flx/flx} MEFs with very small amounts of Cre adenovirus resulted in mixed cultures of *Rce1*^{flx/flx} and *Rce1*^{α/α} MEFs (Bergo et al., 2002a).

To define the effect of the *Rce1* excision on cell growth, we examined *mixed* cultures of *Rce1*^{flx/flx} and *Rce1*^{α/α} cells by transfecting them with large amonts of a *lacZ* adenovirus and small amounts of *Cre* adenovirus (so as to delete *Rce1* from only ~50% of the cells in the culture). The cells were then allowed to grow for multiple passages, and the *Rce1* genotype was checked by Southern blotting at frequent intervals (Bergo et al., 2002a). If the *Rce1* excision had no effect on cell growth (*i.e.,* if there were no differences in the growth of *Rce1*^{flx/flx} and *Rce1*^{α/α} cells), one would expect that the Southern blots would reveal, over multiple passages, stability in the relative intensities of the 5.0-kb *Rce1*^{flx} band and the 6.5-kb *Rce1*^α band. This was not the case. In each of multiple experiments, the ratio of the 5.0-kb *Rce1*^{flx} to the 6.5-kb *Rce1*^α band increased steadily, indicating a competitive growth advantage of the *Rce1*^{flx/flx} cells over *Rce1*^{α/α} cells (Fig. 3). Control experiments established that the competitive growth advantage of *Rce1*^{flx/flx} cells over *Rce1*^{α/α} cells was due to the excision of the *Rce1* gene rather than to the loss of the *neo* (a drug selection marker in the *Rce1*^{flx} allele) (Bergo et al., 2002a).

Effects of an *Rce1* Excision on the Growth of Ras-transfected Cells in Soft Agar and in Nude Mice

In mammalian cells, the expression of activated Ras proteins allow fibroblasts the ability to grow in soft agar (anchorage-independent growth). To test whether a *Rce1* deletion would influence this phenotype, *Rce1*$^{flx/flx}$ fibroblasts were first infected with a retrovirus coding for a mutationally activated K-Ras (Bergo et al., 2002b). After selection for retroviral infection with puromycin (4 μg/ml), the overexpression of K-Ras was documented by western blotting. The Ras-transfected *Rce1*$^{flx/flx}$ fibroblasts were incubated with a *lacZ* adenovirus or a *Cre* adenovirus (to produce Ras-transfected *Rce1*$^{α/α}$ cells) (Bergo et al., 2002a). Equal numbers (3000 cells/well) of Ras-transfected *Rce1*$^{flx/flx}$ or *Rce1*$^{α/α}$ cells were mixed with medium containing 0.35% agarose and poured onto wells of 12-well plates that contained a 0.7% agarose base. The plates were incubated in a humidified culture incubator at 37°C, 7% CO_2, for 14–21 d. Colonies were stained with MTT (3-[4,5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide; thiazolyl blue, Sigma, 1 mg/ml in phosphate-buffered saline). Each well was photographed with a digital camera. The images were imported into Adobe PhotoShop, and colony numbers were determined with an image-processing tool kit, volume 3.0 (http://members.aol.com/ImagProcTK/). The deletion of *Rce1* resulted in a ~30% reduction in colonies in each of four independent experiments ($P = 0.029$; $P = 0.09$; $P = 0.005$; $P = 0.008$) (Bergo et al., 2002b) (Fig. 4). A ~75% reduction in transformed colonies ($P < 0.001$) was observed when using a second pair of *Rce1*$^{flx/flx}$ and *Rce1*$^{α/α}$ cells (not shown). In parallel, we generated H-Ras–transformed *Rce1*$^{flx/flx}$ and *Rce1*$^{α/α}$cells, and found that deletion of *Rce1* resulted in a 65% reduction in colonies ($P = 0.002$). We also obtained K-Ras–transformed *Rce1*$^{−/−}$ and *Rce1*$^{+/+}$ cells by transfecting primary embryonic fibroblasts with E1A and K-Ras. We observed a ~50% reduction in numbers of colonies from *Rce1*$^{−/−}$ cells, compared with *Rce1*$^{+/+}$ cells in three independent experiments ($P = 0.003$, $P < 0.001$, $P < 0.001$) (Bergo et al., 2002a).

The reduced ability of K-Ras–transfected *Rce1*$^{α/α}$ cells to grow in soft agar was associated with a reduced capacity to contribute to the growth of tumors in nude mice (Bergo et al., 2002a). Nude mice were injected with a mixed culture of K-Ras-transfected *Rce1*$^{flx/flx}$ and *Rce1*$^{α/α}$ cells. The *Rce1*$^{flx/flx}$ cells manifested an increased capacity to contribute to the formation of tumors relative to the *Rce1*$^{α/α}$ cell*s.* As judged by Southern blots, the ratio of *Rce1*^{α} to *Rce1*^{flx} alleles in the injected cells was 1.99. The ratio increased to 11.8 ± 2.9 (mean $\pm$ SD) in the DNA prepared from established tumors, reflecting more rapid growth of *Rce1*$^{flx/flx}$ cells relative to *Rce1*$^{α/α}$ cells.

A *Rce1*$^{flx/flx}$ skin carcinoma cell line harboring a mutationally activated H-Ras was produced by treating the skin of *Rce1*$^{flx/flx}$ mice with mutagens (Bergo et al., 2002a). We treated the *Rce1*$^{flx/flx}$ cells with 10^8 pfu of Cre adenovirus, successfully deleting *Rce1* from 90% of the cells. However, we found that the small fraction of *Rce1*$^{flx/flx}$ cells overgrew the culture within a few days in culture, strongly suggesting that *Rce1*$^{flx/flx}$ skin carcinoma cells grow more rapidly than the *Rce1*$^{α/α}$ cells.

Minimal Effects of *Rce1* Deficiency on the Hematopoietic System in Mice

Initially, we considered the possibility that *Rce1*$^{−/−}$ embryos might die from defective hematopoiesis. However, this did not appear to be the case. Lethally irradiated mice were successfully rescued with hematopoietic stem cells from the livers of *Rce1*$^{−/−}$ embryos (Kim et al., 1999). Both the red and white cell counts remained stable for more than 6 months of follow-up, suggesting that there was no significant impact of *Rce1* deficiency on hematopoiesis. ERK activities were measured in bone marrow collected 3 to 6 months after

adoptive transfer. In multiple experiments, *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ bone marrow demonstrated equivalent basal and GM-CSF-stimulated ERK activities.

We considered the possibility that the potential of *Rce1*$^{-/-}$ stem cells might have a *relatively* reduced capacity to repopulate the bone marrow when compared directly with stem cells from *Rce1*$^{+/+}$ bone marrow. However, this was not the case (Aiyagari et al., 2003). Wild-type or *Rce1*$^{-/-}$ fetal liver cells were injected into irradiated hosts with the same reference population of BoyJ competitor cells to directly compare their potential to repopulate the bone marrow. The *Rce1*$^{-/-}$ and *Rce1*$^{+/+}$ hematopoietic cells exhibited equivalent bone-marrow repopulating potential over a dose range that produced 10% to 70% donor cell chimerism. These data strongly suggest that inactivation of *Rce1* does not impair the proliferative capacity of hematopoietic cells.

Analyzing the Functional Importance of *Rce1* in the Liver and Heart

To inactivate *Rce1* in the liver, *Rce1*$^{flx/flx}$ mice were given an intravenous injection of a *Cre* adenovirus (2 α 10^{11} plaque-forming units). After 3 days, livers were removed, and the levels of *CAAX* endoprotease activity in the tissues were determined, and the electrophoretic mobilities of the Ras proteins were analyzed (Bergo et al., 2004). Western blot analysis of the Ras proteins in the liver revealed that approximately one-half of the Ras proteins had retarded electrophoretic mobility, indicating a loss of endoproteolytic processing in a large fraction of the liver cells. Despite the loss of *Rce1* in the liver, the mice remained quite healthy. Mice lacking *Rce1* in the liver were also generated by breeding *Rce1*$^{flx/flx}$ mice carrying the interferon-inducible Mx1-*Cre* transgene, and then inducing *Cre* expression with intraperitoneal injections of polyinosinic-polycytidylic ribonucleic acid (pI-pC, Sigma, St. Louis MO). As judged by Southern blots, the excision of *Rce1* in the livers of the pI-pC-treated mice was complete, and Rce1 activity levels were dramatically reduced. Again, despite the absence of *Rce1* in the liver, the pI-pC-treated *Rce1*$^{flx/flx}$Mx1-*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 and eosin-stained sections was indistinguishable from that of wild-type mice (Bergo et al., 2004).

Mice lacking *Rce1* in the heart were created by breeding *Rce1*$^{flx/α}$ mice carrying a Cre transgene driven by the α-myosin heavy chain (αMyhc) promoter (Bergo et al., 2004). The cardiac myocytes in these mice would be expected to lack *Rce1* expression in cardiac myocytes. Indeed, quantitative PCR studies revealed lower *Rce1* mRNA levels in the hearts of *Rce1*$^{flx/α}$αMyhc-*Cre*$^{+/o}$ mice than in the hearts of *Rce1*$^{flx/α}$ controls ($P < 0.01$) (Bergo et al., 2004). Similarly, using the coupled endoprotease/methylation assay, we observed lower *CAAX* endoprotease activity levels in the hearts of *Rce1*$^{flx/α}$αMyhc-*Cre*$^{+/o}$ mice than in the hearts of *Rce1*$^{flx/α}$ control mice ($P < 0.01$). As expected, we observed retarded electrophoretic mobility of the Ras proteins in hearts of *Rce1*$^{flx/α}$αMyhc-*Cre*$^{+/o}$ mice, and we were able to document, with the coupled endoprotease/methylation assay, a significant accumulation of Rce1 protein substrates in the hearts of *Rce1*$^{flx/α}$αMyhc-*Cre*$^{+/o}$ mice.

The *Rce1*$^{flx/α}$αMyhc-*Cre*$^{+/o}$ mice and control *Rce1*$^{flx/flx}$ mice were bred and observed for 10 months. The *Rce1*$^{flx/α}$αMyhc-*Cre*$^{+/o}$ mice appeared healthy at 1 month of age, but started dying by 3–5 months of age (Bergo et al., 2004). By 7 months of age, 50% of the *Rce1*$^{flx/α}$αMyhc-*Cre*$^{+/o}$ mice had died; by 10 months, 70% had died. In contrast, none of the *Rce1*$^{flx/flx}$ mice or *Rce1*$^{+/+}$ αMyhc-*Cre*$^{+/o}$ mice died during a 10-month follow-up period. Similarly, no premature deaths were observed in *Rce1*$^{flx/α}$ mice. Several weeks before their deaths, most of the heart-specific *Rce1* knockout mice (*Rce1*$^{+/+}$ αMyhc-*Cre*$^{+/o}$) appeared listless and had ruffled fur. The hearts of *Rce1*$^{+/+}$ αMyhc-*Cre*$^{+/o}$ mice were invariably enlarged (Fig. 5), and echocardiography showed dilated left ventricles (Bergo et al., 2004). Histological sections

revealed dilatation of all four chambers of the heart, and organized thrombi were occasionally noted within the left atrium. The left ventricular musculature was thin, and there were increased amounts of collagen between myocytes. Heart tissue from $Rce1^{flx/flx}$, $Rce1^{+/+}$αMyhc-$Cre^{+/o}$, and $Rce1^{flx/α}$ control mice was normal. Thus, in contrast to the situation with the liver and hematopoietic cells, $Rce1$ deficiency in the heart had significant adverse consequences.

It is tempting to speculate that the effects of $Rce1$ deficiency in the heart are somehow related to the absence of Ras endoproteolytic processing, particularly given that an activated H-Ras transgene in mice causes myocardial hypertrophy (Gottshall et al., 1997) and the expression of a dominant-negative H-Ras causes a dilated cardiomyopathy (Dr. Seigo Izumo, personal communication; see also http://cardiogenomics.med.harvard.edu/groups/proj1/pages/ras_home.html). However, despite the attractiveness of the "Ras hypothesis," there are clearly reasons for caution. First, we did not observe any effect of $Rce1$ deficiency in fibroblasts on the activation of Ras effectors by growth factors (Bergo et al., 2004). Second, Rce1 has dozens of protein substrates, and it is conceivable that the elimination of endoproteolysis of some of the non-Ras substrates underlies the pathology in the heart-specific $Rce1$ knockout mice. For example, the endoproteolytic processing of lamin B1 (a key structural component of the nuclear lamina) does not occur in the absence of $Rce1$ (Maske et al., 2003). Missense mutations in lamin A/C clearly cause dilated cardiomyopathy (Fatkin et al., 1999), so one could imagine that absent endoproteolytic processing of lamin B1 might affect heart function. One could make similar arguments about the potential involvement of many other $CAAX$ proteins in mediating the cardiomyopathy of $Rce1$ deficiency.

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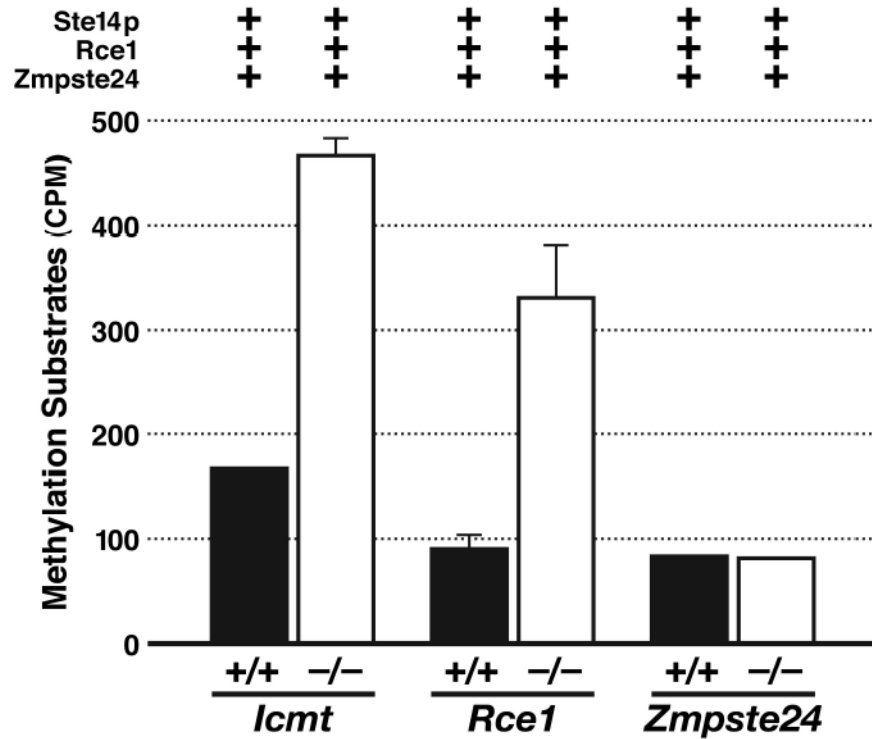
Fig 1.
An accumulation of "methylatable" substrates in *Rce1* $^{-/-}$ cells. Methylation of whole-cell extracts from primary fibroblasts derived from *Zmpste24*$^{+/+}$, *Zmpste24* $^{-/-}$, *Icmt*$^{+/+}$, *Icmt* $^{-/-}$, *Rce1*$^{+/+}$, *and Rce1* $^{-/-}$ embryos. The cell extracts were incubated with *S*-adenosyl-ʟ-[*methyl*-^{14}C]methionine, membranes containing high levels of Ste14p, yeast membranes containing high levels of mouse Rce1, and yeast membranes containing high levels of mouse Zmpste24. The relative level of methylatable substrates in wild-type and knockout cells was assessed with a base hydrolysis/methanol diffusion assay. For the *Zmpste24*$^{+/+}$ and *Zmpste24* $^{-/-}$ cells, we observed identical results when membranes expressing Rce1 were left out of the reaction mixture. Reproduced, with permission from *The Journal of Biological Chemistry* (Leung et al., 2001).
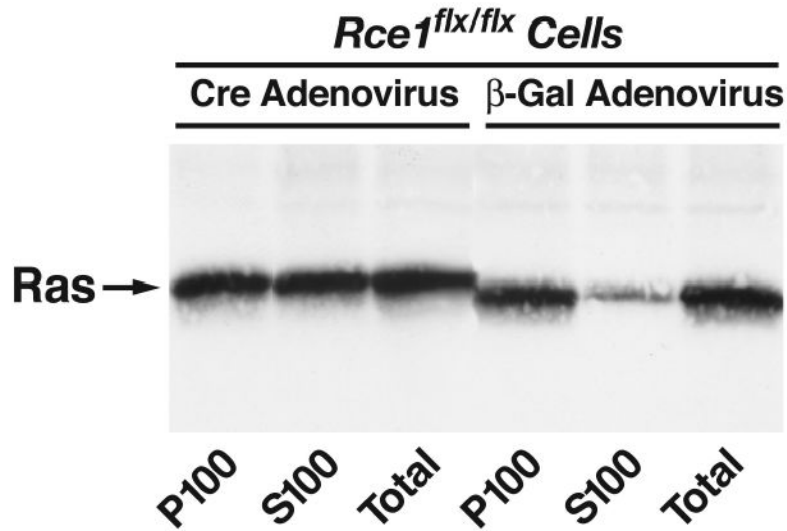
Fig 2.
Intracellular localization of Ras proteins in immortalized fibroblasts. *Rce1* flx/flx cells express normal levels of *Rce1*, whereas *Rce1* α/α fibroblasts are completely deficient in *Rce1* expression. *Cre* adenovirus fully converted the *Rce1*flx/flx fibroblasts into *Rce1* α/α fibroblasts. Fibroblasts were fractionated into cytosolic (S100) and membrane (P100) fractions; Ras proteins were immunoprecipitated and analyzed on western blots of sodium dodecyl sulfate-polyacrylamide gels with antibody Ab-4. Note higher molecular weight of the Ras proteins in *Rce1* α/α fibroblasts. Reproduced, with permission from *Molecular and Cellular Biology* (Bergo et al., 2002a).
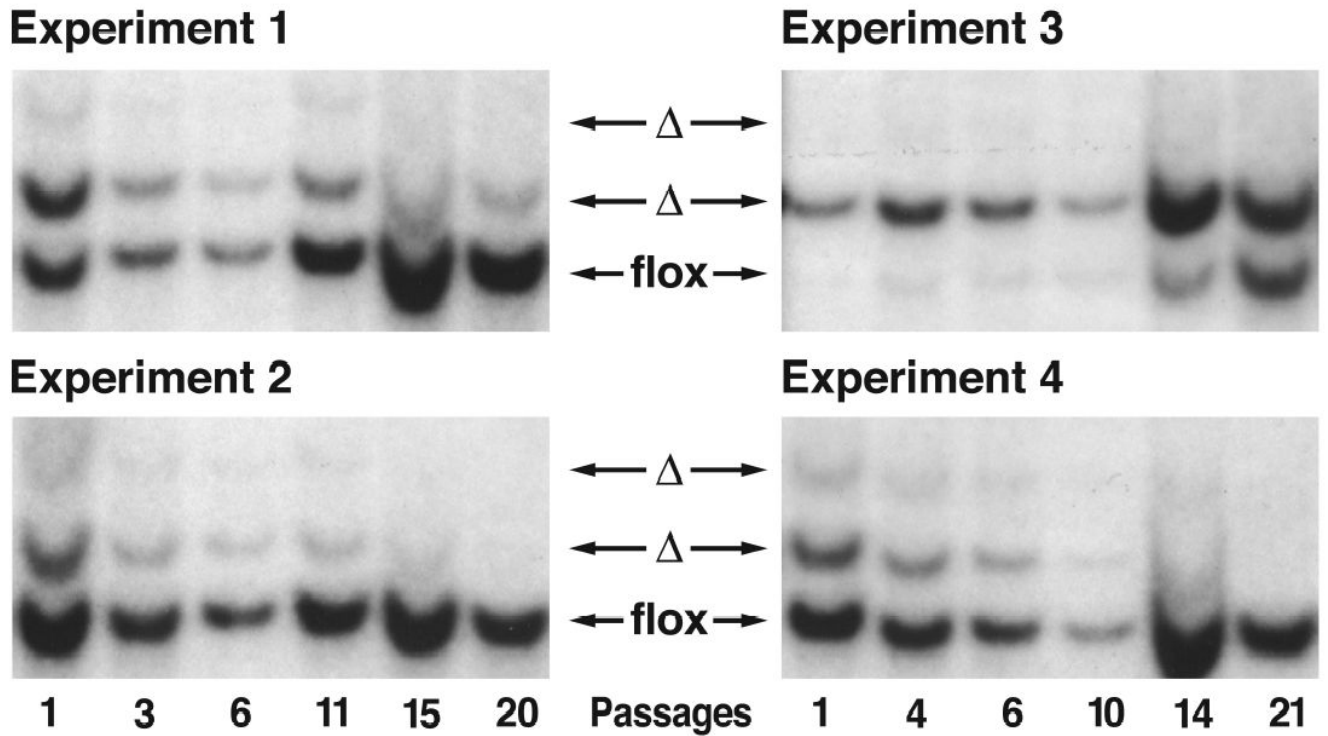
Fig 3.
Southern blot assessment of the ratio of *Rce1*^{flx} and *Rce1*$^{\alpha}$ alleles during the growth of mixed cultures of *Rce1*$^{flx/flx}$ and *Rce1*$^{\alpha/\alpha}$ fibroblasts. Mixed cultures of *Rce1* $^{flx/flx}$ and *Rce1*$^{\alpha/\alpha}$ cells were obtained by infecting immortalized *Rce1*$^{flx/flx}$ fibroblasts with *lacZ* adenovirus and *Cre* adenovirus. Southern blots showing the ratio of *Rce1*$^{\alpha}$ and *Rce1*^{flx} alleles at different passages in four independent experiments. These experiments show that the *Rce1*$^{flx/flx}$ cells, which express *Rce1*, grow more rapidly than the *Rce1*$^{\alpha/\alpha}$ fibroblasts, which lack *Rce1* expression. Reproduced, with permission from *Molecular and Cellular Biology* (Bergo et al., 2002a).
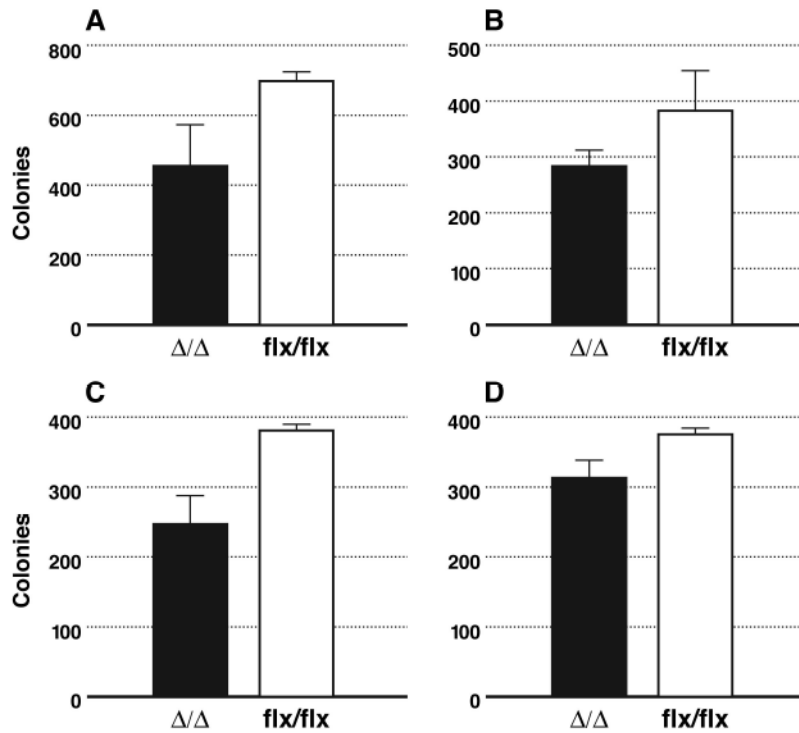
Fig 4.
Comparison of the ability of K-Ras–transformed immortalized *Rce1*$^{\alpha/\alpha}$ and *Rce1*$^{\text{flx/flx}}$ fibroblasts to form colonies in soft agar. *Rce1*$^{\text{flx/flx}}$ fibroblasts were infected with a K-Ras retrovirus, then treated with either *lacZ* adenovirus or *Cre* adenovirus. In *Rce1*$^{\text{flx/flx}}$ cells treated with *Cre* adenovirus, the *Rce1* was fully deleted, generating *Rce1*$^{\alpha/\alpha}$ fibroblasts. In each experiment, the excision of *Rce1* resulted in fewer colonies in soft agar ($P = 0.029$, $P = 0.005$; $P = 0.09$; $P = 0.008$). Reproduced, with permission from *Molecular and Cellular Biology* (Bergo et al., 2002a).
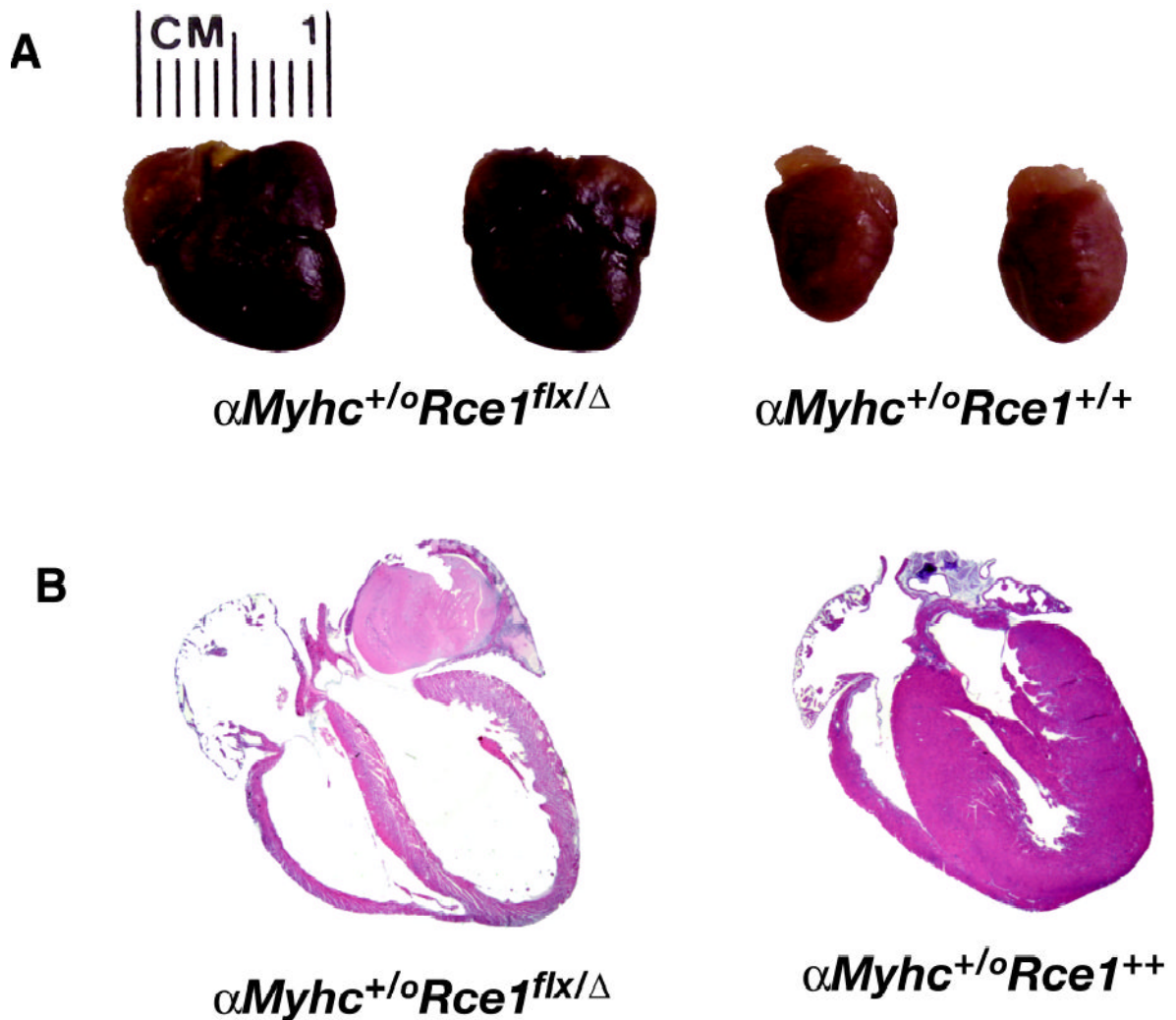
Fig 5.
Dilated cardiomyopathy in *Rce1* lx/α αMyhc-*Cre*+/o mice. *A*, Increased size of hearts from *Rce1*flx/α αMyhc-*Cre*+/o mice, compared with *Rce1*+/+αMyhc-*Cre*+/o controls. B, H&E-stained sections of an *Rce1*flx/α αMyhc-*Cre*+/o heart and an *Rce1*+/+αMyhc-*Cre*+/o heart, both from 7-month-old mice. The hearts from *Rce1*flx/α αMyhc-*Cre*+/o mice were invariably dilated. Note the organized left atrial thrombus (arrow) in the heart from the *Rce1*flx/α αMyhc-*Cre*+/o mouse. Reproduced, with permission from *The Journal of Biological Chemistry* (Bergo et al., 2004).