Biochemical Studies of Zmpste24-deficient Mice*

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Genetic studies in Saccharomyces cerevisiae identified two genes, STE24 and RCE1, involved in cleaving the three carboxyl-terminal amino acids from isoprenylated proteins that terminate with a CAAX sequence motif. Ste24p cleaves the carboxyl-terminal “-AAX” from the yeast mating pheromone a-factor, whereas Rce1p cleaves the -AAX from both a-factor and Ras2p. Ste24p also cleaves the amino terminus of a-factor. The mouse genome contains orthologues for both yeast RCE1 and STE24. We previously demonstrated, with a gene-knockout experiment, that mouse Rce1 is essential for development and that Rce1 is entirely responsible for the carboxyl-terminal endoproteolytic processing of the mouse Ras proteins. In this study, we cloned mouse Zmpste24, the orthologue for yeast STE24 and showed that it could promote a-factor production when expressed in yeast. Then, to assess the importance of Zmpste24 in development, we generated Zmpste24-deficient mice. Unlike the Rce1 knockout mice, Zmpste24-deficient mice survived development and were fertile. Since no natural substrates for mammalian Zmpste24 have been identified, yeast a-factor was used as a surrogate substrate to investigate the biochemical activities in membranes from the cells and tissues of Zmpste24-deficient mice. We demonstrated that Zmpste24-deficient mouse membranes, like Ste24p-deficient yeast membranes, have diminished CAAX proteolytic activity and lack the ability to cleave the amino terminus of the a-factor precursor. Thus, both enzymatic activities of yeast Ste24p are conserved in mouse Zmpste24, but these enzymatic activities are not essential for mouse development or for fertility.

Proteins that terminate in a carboxyl-terminal CAAX motif undergo three sequential enzymatic processing events, farnesylation or geranylgeranylation of the cysteine, endoproteolytic release of the last three amino acid residues of the protein (i.e. removal of the -AAX), and methylation of the new carboxyl terminus of the protein by isoprenylethylamine carboxyl methyltransferase (1, 2). The yeast genes responsible for the farnesylation and methylation steps were identified more than a decade ago (3, 4), but the identification of the genes responsible for the middle processing step, the endoprotease step, remained elusive for years (2). Ultimately, however, Boyartchuk and co-workers (5) applied a novel genetic selection scheme and identified two yeast genes, RCE1 and STE24 (AFC1), involved in the carboxyl-terminal endoproteolytic processing of isoprenylated CAAX proteins. Rce1p is a protease involved in the carboxyl-terminal processing of both a-factor and the yeast Ras protein, Ras2p. Ste24p (Afclp), a zinc metalloprotease, lacks activity against Ras2p but did process a-factor. Haploid MATa yeast lacking both RCE1 and STE24 (ste24Δrce1Δ) grew normally but were unable to produce mature a-factor and therefore were sterile (5). Interestingly, Rce1p and Ste24p exhibited subtle differences in substrate specificities (5–7). Both proteins were capable of cleaving the carboxyl terminus of wild-type a-factor, which terminates in CVIA. However, only Ste24p processed an a-factor mutant terminating in CAMQ, and only Rce1p processed an a-factor mutant terminating in CTLM.

The identification of Ste24p as a CAAX endopeptidase was initially surprising because Ste24p had also been described as a protease that cleaves the amino terminus of the a-factor precursor protein (8), and it seemed improbable that a single protease would recognize and cleave a single protein at two completely different sites. Subsequent studies, however, established that Ste24p does indeed play dual roles in a-factor processing (6, 9). In addition to being a CAAX endopeptidase, Ste24p removes the seven amino-terminal amino acids from the a-factor precursor. Ste24 is, however, only the first of two proteases that cleave the amino terminus of a-factor. After the Ste24-mediated cleavage step, an additional cleavage by Ax1lp releases mature biologically active a-factor.

When the two CAAX endopeptidases were identified in yeast, apparent orthologues already existed in mammalian cDNA data bases. Tam and co-workers (9) obtained a full-length cDNA for human ZMPSTE24, the orthologue for yeast STE24, and showed that its amino acid sequence was 36% identical to the yeast protein. No a-factor orthologue has been discovered in mammals, and no natural substrates for human ZMPSTE24 have been identified. However, the human enzyme faithfully carried out both the amino-terminal and carboxyl-terminal processing of a surrogate substrate, yeast a-factor, and restored a-factor production in MATa ste24Δrce1Δ yeast (9, 10).
Our laboratory has sought to define the physiologic importance of the mammalian CAAX endoproteases, as well as the CAAX methyltransferase, isoprenylcysteine carboxyl methyltransferase (ICMT). We cloned and expressed cDNAs for human and mouse Rce1 and showed that the mammalian Rce1 proteins processed the Ras proteins as well as several other CAAX proteins (11, 12). Rce1 knockout mice died during embryonic development (11). However, studies with Rce1-deficient embryos and embryonic fibroblasts showed that Rce1 is essential for Ras processing (11). Like Rce1, ICMT is essential for the processing of mammalian CAAX proteins and also for survival during embryonic development (13).

In the current study, we have turned our attention to cloning mouse Zmpste24 and producing Zmpste24-deficient mice. When we initiated this project, we had several physiological and biochemical issues in mind. First, we wanted to gauge the physiologic importance of Zmpste24 in mammals. Would Zmpste24 be required for embryonic development, as was the case for Rce1 and ICMT? If Zmpste24-deficient mice survived, would they be fertile? From the biochemical perspective, we wanted to determine whether mouse Zmpste24 would be capable of carrying out the carboxyl-terminal processing of a factor and, if so, whether the mouse enzyme would manifest the same peculiar specificities as the yeast enzyme (e.g. the ability to cleave an a-factor mutant terminating in CAMQ). We also wanted to determine whether the mouse enzyme would cleave the amino terminus of yeast a-factor and, if so, whether this enzymatic activity would be present in detectable levels in the tissues of wild-type mice. Finally, we wanted to determine if mouse Zmpste24 was the only mammalian enzyme capable of carrying out the amino-terminal processing of an a-factor, or whether mammalian cells might contain more than one enzyme with that activity. Our current studies provide insights into each of these issues.

EXPERIMENTAL PROCEDURES

A Mouse Zmpste24 cDNA Clone—A XREFDb query (14) of GenBank® sequences with yeast STE24 sequences identified a human expression screening clone (M.A.G.E. clone 284572, Research Genetics, Huntsville, AL). Oligonucleotide primers 5'-GAGTTTCAACGTGATCGTTGC-3' and 5'-GAGTTTTCAACGTGATCGTTGC-3' derived from the human sequence were used to amplify a 382-base pair (bp) fragment (representing sequences from Zmpste24 exons 9 and 10) from a mouse liver cDNA library. The 5' portion of the mouse Zmpste24 cDNA was generated by 5' rapid amplification of cDNA ends with oligonucleotide 5'-CTCTTTACGCTTTCCAACGTTC-GTGC-3' (corresponding to Zmpste24 amino acids 422-430) and the Marathon-Ready mouse liver cDNA kit (CLONTECH, Palo Alto, CA). Oligonucleotides 5'-ACTCTCCAGGAGCCGCGTG-3' and 5'-CTTAA-GAGCTACGACATT-3' were used to amplify a mouse Zmpste24 cDNA. The cDNA was cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA), and both strands were sequenced. Predicted transmembrane domains were determined with the TMHMM transmembrane domain analysis program (genome.cbs.dtu.dk/services/TMHMM-1.0/). Amino acid sequences from different species were aligned with MacVector 6.5.

Expression of Zmpste24 in different mouse tissues was assessed by hybridizing a 32P-labeled Zmpste24 cDNA probe to a mouse Multiple Choice Northern blot (OrigiGene Technologies, Rockville, MD) and to a mouse multiple-tissue poly(A) RNA blot (CLONTECH) and to a mouse liver cDNA (CLONTECH) with oligonucleotides 5'-CATCTATTACATGTAATACCAATGCTTAGTAA-TCTTGAATAAATTCCAGAGATT-3' and 5'-TTGATCTATCGATTTCAATTCAATT-3' (corresponding to Zmpste24 amino acids 64-79). The ability of each expression vector to restore yeast a-factor production was assessed in yeast strain W303-1a, which has the ADH2 and PKR1 products, and Yeplac195 which had been linearized with HindIII and EcoRI.

All expression vectors were created by recombinatorial cloning after co-transformation of XbaI-cleaved pMB1 and PCR-amplified endoprotease open reading frames. A mouse Zmpste24 open reading frame was amplified with oligonucleotides 5'-CTATAAATATGCTAGTGACC-3' and 5'-CTGAGTCGAGCGGCTGG-3' and was sequenced. A mutant Zmpste24 cDNA (pMB9) was also constructed; the mutant lacks the sequences from the 104-bp exon 8, which encodes the zinc-binding HEXXH motif (HEXXH: H, histidine; E, glutamate; X, any amino acid). For this mutant, two fragments of the mouse Zmpste24 cDNA (corresponding to amino acids 1-318 and 345-475) were amplified from Marathon Ready mouse liver cDNA (CLONTECH) with oligonucleotides 5'-CTATAAATATGCTAGTGACC-3', 5'-CTATAAATATGCTAGTGACC-3', and 5'-TGGTCTATCGATTTCAATTCAATT-3'. To construct pMB5, a yeast STE24 (YJR111W) open reading frame was amplified from W303-1a genomic DNA with oligonucleotides 5'-CAACTTAATATCATGTAATACCA-ATGTTCTATCGATTTCAATT-3' and 5'-TTGATCTATCGATTTCAATT-3'. The ability of yeast and mouse endoproteases to process 15-mer CAAX-box in the mouse Zmpste24 cDNA was assessed in a coupled proteolysis/methylation reaction with Zmpste24-deficient mice.

Analyzing the Substrate Specificity of Mammalian Endoproteases with Halo Assays—The coding regions of human Rce1 and mouse Zmpste24 were amplified by PCR, as described earlier, and cloned into pACA1 (5). Site-directed mutagenesis was used to change the CAAX box in the a-factor gene (MPA1) to CAMQ or CTTM. The mutant a-factor plasmids were transformed into a yeast strain (YJR5463) (6). Yeast strains expressing the a-factor mutants and either human RCE1 or mouse Zmpste24 were spotted onto a lawn of yeast sst2 cells (YJR3443) and allowed to grow for 2 days at 30 °C. The production of mature a-factor by a yeast strain results in a zone of growth inhibition (halo) on a lawn of Yeast sst2 cells.

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Construction of a Zmpste24 Gene-Targeting Vector—The screening of a strain 129/Sv bacterial artificial chromosome (BAC) library (Research Genetics) with the 382-bp cDNA fragment resulted in the identification of a BAC clone (223 G6) that contained the 3′ portion of mouse Zmpste24. A 9.4-kb SpeI fragment containing Zmpste24 exons 7–9 was subcloned into pBSK (Stratagene, La Jolla, CA). These sequences were used to construct a sequence-replacement gene-targeting vector in pKSLoxP NT. pKSLoxP NT contains polyclinker cloning sites, a thymidine kinase (tk) gene, and a 3-phosphoglycerate kinase-neomycin resistance (PGK-neo) cassette flanked by loxP sites. The long arm (4.6 kb of intron sequences 5′ to exon 8) was amplified from BAC DNA with oligonucleotides 5′-CAAGATGTTGAGCTGCCTCGT-3′ and 5′-GGCTTC- TACCAAGACACACAG-3′ and the Expand Long Template PCR System (Roche Molecular Biochemicals, Indianapolis, IN). The PCR fragment was cloned into the EcoRI site of pKSLoxP NT. The short arm (2.6 kb of intron sequences 3′ to exon 8) was amplified with oligonucleotides 5′-ACGCCGGGCGCGGAAATCTGCCTGTTGCTTACTATGAC-3′ and 5′-AGCACCAGCGCGGGAGAACAACACAACAATGCATTGACAG-3′ and cloned into the NotI site. The integrity of the vector was verified by restriction mapping and DNA sequencing. The vector was linearized with XhoI before the electroporation of mouse embryonic stem (ES) cells.

Generation of Zmpste24-deficient Mice—Mouse ES cells (strain 129/SvJae) (24) were cultured on mitomycin C-treated STO feeder cells in high-glucose Dulbecco's modified Eagle's medium supplemented with 15% ES cell-grade fetal bovine serum, 2 mM L-glutamine, 6 mM HEPES, 1.5 mM sodium pyruvate, 1 mM sodium bicarbonate, 55 mM NaHCO3, 100 mM NaCl, 30 mM HEPES, 100 mM NaHPO4, and 200 mM NaCHO3 containing 1% DMSO and 1% penicillin, streptomycin, and fungizone, at 37 °C. The methylation reaction was stopped by adding 50 mM NaOH containing 0.1% SDS. The reaction mixture (90 μl) was spotted onto a 2 × 8 cm filter paper, washed with water, and then placed in the neck of a 20-ml scintillation vial containing 5 ml of scintillation fluid (ScintiSafe Econo 1, Fisher). The vials were capped and incubated at room temperature for 5 h to allow the [3H]methanol (formed by base-hydrolysis of methyl esters) to diffuse into the scintillation fluid (20–22).

Assessing the Ability of Mouse Membranes to Carry Out the Amino-terminal Processing of a Factor—Yeast membranes containing radiolabeled P1 a-factor were prepared as previously described (10). P1 a-factor is fully COOH-terminal modified (i.e. isoprenylated, cleaved, and carboxyl methylated) (29). In a wild-type strain, COOH-terminal processing of a-factor occurs first and is followed by NH2-terminal processing (29). Isoprenylation is required (29) for the production of P1 a-factor, but blocking either methylation (30) or CAAX processing does not impede NH2-terminal processing. Briefly, a yeast strain lacking STE42 but expressing MFA1 from a high-copy plasmid (SM3103/pSM219) was radiolabeled with [35S]methionine and membranes containing P1 a-factor were isolated (10). Proteolysis reactions (typically 50 μl) were conducted on whole or membrane preparations using 96-well plastic plates. The reactions were assembled in 96-well polystyrene plates in a total volume of 25 μl. After a 2-h incubation at 30 °C, the reactions were terminated by adding copper acetate (final concentration, 2 mM). The production of mature a-factor was detected by plating dilutions of the reaction mixture on a lawn of MA-TAT2 ES cells (SM1086) and allowing the cells to grow for 2 days at 30 °C (27, 28). Mature a-factor results are shown in zone of growth in the lawn of cells.

Assessing the Accumulation of "Methylatable" Substrates in Rec1-, ICMT-, and Zmpste24-deficient Fibroblasts—Whole cell extracts from primary fibroblasts (Zmpste24+/+, Zmpste24−/−, ICMT+/+, ICMT−/−, Rec1+/+, Rec1−/−) (11, 13) were incubated with 10 μM S-adenosyl-l-[methyl-14C]methionine (55 Ci/mol, Amersham Pharmacia Biotech), 0.5 mg/ml leupeptin, chymostatin, and pepstatin) at concentrations of 13–14 mg/ml and then frozen at −80 °C. A 100 μl solution of the CAMQ peptide was prepared in methanol. Membranes from ste24Δ/rec1Δ yeast that expressed STE14 from a 2-μm plasmid (SM3614/pSM1317) were prepared as a source of the prenylprotein methyltransferase (15). Each carboxyl-terminal processing reaction contained the a-factor peptide (0.75 μmol), membranes from mouse tissues (75 μg/ml), ste24Δ/rec1ΔSte14 yeast membranes (0.1 mg/ml) in a buffer containing 75 mM HEPES, 75 mM NaCl, 15 mM EDTA, pH 7.5, 250 mM NaF, 100 mM Na2SO4, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 3 μg/ml leupeptin, chymostatin, and pepstatin) at concentrations of 3–14 mg/ml and then frozen at −80 °C. A 100 μl solution of the CAMQ peptide was prepared in methanol.

Bioassays to Assess Carboxyl-terminal Processing of an a-Factor Peptide by Membranes from Mouse Cells and Tissues—The ability of Zmpste24−/− membranes to cleave the carboxyl terminus of an a-factor peptide, generating mature a-factor, was assessed by a bioassay.3 Membranes from tissues of Zmpste24+/+ and Zmpste24−/− mice and from Zmpste24+/+ and Zmpste24−/− cells were mechanically disrupted by sonication (Branson sonifier, 10 cycles, output = 5, duty cycle 50%) in an ice-cold hypoxanthine buffer (10 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 3 μg/ml leupeptin, chymostatin, and pepstatin). The samples were then homogenized on ice with a Dounce homogenizer, resuspended in 0.15 M NaCl, and then subjected to centrifugation at 4 °C for 3000 × g for 10 min. The pellet was discarded and the supernatant fluid was spun at 100,000 × g for 1 h. The supernatant fluid was discarded and the pellet, representing the membrane fraction, was resuspended in ice-cold buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 1 mM EDTA, 100 mM NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 3 μg/ml leupeptin, chymostatin, and pepstatin) at concentrations of 3–14 mg/ml and then frozen at −80 °C.

3 T. A. Tam, W. K. Schmidt, and S. Michaelis, manuscript in preparation.

4 W. K. Schmidt and S. Michaelis, unpublished data.
base hydrolysis/methanol diffusion assay. All incubations were performed in duplicate.

**RESULTS**

A Mouse Zmpste24 cDNA—DNA sequencing of a mouse Zmpste24 cDNA revealed that the mouse enzyme, like the human enzyme, contains 475 amino acids (Fig. 1). The mouse and human amino acid sequences were 93% identical. Both the mouse and human sequences were 36% identical to the *S. cerevisiae* Ste24p sequence, with the highest levels of similarity within the last third of the molecule. Mouse Zmpste24, like the human and yeast enzymes, has a HE\textsuperscript{XX}H motif (residues 335–339) that is characteristic of zinc metalloproteinases (5, 8, 9) and multiple membrane-spanning domains (Fig. 1). Mouse Zmpste24 is expressed in multiple tissues, with the highest levels of expression in the liver and kidney (Fig. 2).

Functional Studies of Mouse Zmpste24 in Yeast—To assess the functional integrity of mouse Zmpste24, a yeast plasmid encoding the Zmpste24 cDNA was transformed into ste24\textsuperscript{Δ}rce1\textsuperscript{Δ} yeast. The transformed cells were tested for the ability to produce α-factor with an established bioassay that utilizes a lawn of *MATa sst2* cells. Nontransformed ste24\textsuperscript{Δ}rce1\textsuperscript{Δ} yeast do not produce a zone of growth inhibition (halo) on the lawn of *MATa sst2* cells since they cannot form mature α-factor. The plasmid encoding mouse Zmpste24 restored α-factor production, as judged by the halo surrounding the colony (Fig. 3A). Mouse Rce1 also restored α-factor production (Fig. 3A). In anticipation of performing a gene-targeting experiment in mice, we sought to establish that the removal of the 104-bp exon 8 of Zmpste24 (encoding amino acids 318–354, containing the HEXXH motif) would inactivate the gene product. We therefore transformed the ste24\textsuperscript{Δ}rce1\textsuperscript{Δ} yeast with a mutant mouse Zmpste24 cDNA lacking exon 8 sequences. The mutant cDNA did not restore α-factor production (Fig. 3A).

Boyartchuk and co-workers (6) demonstrated that yeast Ste24p and Rce1p have distinct specificities for α-factor precursors with different CAAX sequences. Both yeast Ste24p and yeast Rce1p processed the wild-type α-factor precursor, which terminates in CVIA (5, 6). Ste24p was uniquely capable of processing mutant α-factor precursor molecules terminating in CAMQ, while Rce1p was uniquely capable of cleaving a mutant α-factor terminating in CTLM. Interestingly, we found that the mammalian enzymes exhibited the same specificities. Both mouse Rce1 and mouse Zmpste24 were capable of processing the wild-type α-factor terminating in CVIA (Fig. 3A). However, only RCE1 processed a mutant α-factor terminating in CTLM, and only Zmpste24 processed a mutant α-factor terminating in CAMQ (Fig. 3B).

The fact that both yeast Ste24p and mouse Zmpste24 can cleave a CAMQ α-factor was confirmed with coupled endoproteolysis/methylation assays (Fig. 3C). Membranes from ste24\textsuperscript{Δ}rce1\textsuperscript{Δ} yeast that overexpressed yeast STE24 or mouse Zmpste24 cleaved and methylated a CAMQ α-factor peptide.

**FIG. 1.** The mouse Zmpste24 amino acid sequence, aligned with the human and yeast sequences. A conserved HEXXH zinc-binding motif is present in each of the three sequences (residues 335–339 of the mouse and human sequence). The mouse sequence is predicted to contain multiple transmembrane domains (residues 19–37, 80–102, 121–143, 171–189, 198–216, 349–367, and 386–408). The yeast and human sequences also are predicted to contain multiple transmembrane domains (2). GenBank\textsuperscript{TM} for mouse Zmpste24 cDNA sequence: AY029194.

**FIG. 2.** Northern blot showing Zmpste24 expression in multiple mouse tissues. A \textsuperscript{32}P-labeled Zmpste24 cDNA probe was hybridized to a mouse Multiple Choice Northern blot (OriGene). Loading of samples was normalized to β-actin. The blot was exposed to x-ray film for 72 h at –80 °C. A similar pattern was observed with a multiple tissue poly(A)\textsuperscript{+} RNA blot from CLONTECH (not shown).
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Yeast membranes overexpressing the mutant mouse Zmpste24 (lacking the HXXH motif) did not methylate the peptide, nor did yeast membranes that overexpressed yeast Rce1p or mouse Rce1. In contrast, membranes from yeast expressing mouse Zmpste24, yeast STE24, mouse Rce1, and yeast RCE1 cleaved and then methylated the wild-type CVIA a-factor peptide (Fig. 3D).

Zmpste24 Knockout Mice—A gene-targeting vector designed to replace exon 8 of Zmpste24 with a neo was used to inactivate mouse Zmpste24 (Fig. 4A). Approximately 1 in 30 drug-resistant clones was targeted (Fig. 4B). Two ES cell lines were used to generate Zmpste24+/− mice, which were bred to generate Zmpste24−/− mice (Fig. 4C). Zmpste24−/− mice (produced from both targeted ES cell clones) were born at the expected mendelian frequency and were viable and fertile. To assess the relative capacities of wild-type cells and Zmpste24−/− cells to contribute to the formation of different mouse tissues, chimeric mice were generated with Zmpste24−/− ES cells (Fig. 4D). A total of 12 high-percentage (>90%) chimeric mice were obtained. As judged by Southern blot analysis, the Zmpste24-deficient ES cells contributed robustly to the formation of each tissue that was analyzed (heart, lung, liver, spleen, kidney, adipose, brain, intestine, skin, testes, and skeletal muscle) (not shown).

Assessing Zmpste24 Enzymatic Activity Levels—We next sought to determine if the targeted mutation in Zmpste24−/− mice was associated with detectable changes in enzymatic activity levels. Since no natural substrates for mammalian Zmpste24 have been described, we tested the possibility that the membranes from Zmpste24−/− cells and tissues would be deficient in their capacity to process a surrogate substrate, yeast a-factor. In the first series of experiments, we incubated Zmpste24−/− and Zmpste24+/+ membranes with the CAMQ a-factor peptide in the presence of [S-adenosyl-L-[methyl-14C]methionine, and then assessed base-labile methylation of the peptide. Similar levels of methylation were observed with both Zmpste24−/− and Zmpste24+/+ membranes (Zmpste24+/+, 0.82 ± 0.02 pmol/mg/min; Zmpste24−/−, 0.78 ± 0.02 pmol/mg/min), and the methylation was sensitive to 1,10-phenanthroline (data not shown). This result was clearly different from what we observed with the coupled endoprotease/methylation assays involving yeast membranes; Zmpste24-containing yeast membranes processed the CAMQ a-factor peptide significantly more than membranes lacking Zmpste24 (see Fig. 3C).

We suspected that the failure to find differences in methylation of the CAMQ a-factor peptide by Zmpste24−/− and Zmpste24+/+ membranes was due to the presence of a membrane-bound, phenanthroline-sensitive exoprotease in the mouse membranes, which would be expected to generate farnesylcysteines or short farnesylcysteine-containing peptides. The latter substances would be readily methylated and would increase the background in the experiment, making it impossible to detect potential differences between the ability of Zmpste24−/− and Zmpste24+/+ membranes to carry out the carboxyl-terminal endoproteolytic processing of a-factor. To circumvent this problem, we decided to perform a more specific assay, a yeast bioassay for fully processed mature a-factor. In this assay, we tested whether membranes from Zmpste24-deficient cells and tissues would be defective in producing mature a-factor from the 15-mer CAMQ a-factor peptide (i.e. defective in cleaving the -AMQ from the peptide, making it a substrate for carboxyl methylation). To address this issue, the CAMQ a-factor peptide was mixed with mouse liver membranes for 2 h, and the production of a-factor was assessed by assessing the ability of mature a-factor to inhibit the growth of MATα sst2 yeast. Notably, membranes from livers of Zmpste24−/− mice were 4–8 times less efficient in producing mature a-factor (Fig. 5A). Similar results were observed in other tissues in an independent experiment (Fig. 5B).
We also examined the possibility that membranes from Zmpste24−/− cells and tissues would be deficient in their ability to cleave the amino terminus of a factor intermediate. In yeast, both in vitro and in vivo, Ste24p cleaves the seven amino-terminal amino acids from P1 (farnesylated factor precursor), generating a shorter intermediate, P2, which can be resolved from P1 by SDS-PAGE (9, 10, 29). We produced a metabolically labeled P1 intermediate in ste24Δ yeast, then tested the ability of Zmpste24−/− and Zmpste24+/+ membranes to cleave the amino terminus from P1, converting it to P2 (Fig. 6). Membranes from Zmpste24+/+ ES cells and fibroblasts converted P1 to P2, and this activity was blocked by 1,10-phenanthroline (Fig. 6A). Membranes from Zmpste24−/− ES cells and fibroblasts lacked this activity (Fig. 6A). Similarly, membranes from the liver, heart, and skeletal muscle of Zmpste24−/− mice converted P1 to P2, whereas membranes from Zmpste24−/− mice did not (Fig. 6B).

Testing for the Accumulation of Substrates in Rce1−/− and Zmpste24−/− Cells—Although no natural Zmpste24 CAAX protein substrates have been identified, the high degree of sequence conservation in the enzyme suggests that natural substrates must exist. We hypothesized that significant amounts of CAAX protein substrates might accumulate in both Rce1-deficient cells and Zmpste24-deficient cells. To test that possibility, whole cell extracts from primary fibroblasts (Zmpste24−/+, Zmpste24−/−, Rce1+/+, and Rce1−/−) were incubated with [3H]farnesyl-[methionyl]14C]methionine and membranes containing high levels of Ste14p, mouse Rce1, and mouse Zmpste24. As a control, we examined the accumulation of protein substrates in whole cell extracts from ICMT−/+ and ICMT−/− fibroblasts (13). The accumulation of methylatable substrates in each extract was gauged with a base hydrolysis/methanol diffusion assay. As expected, methylatable substrates accumulated in ICMT−/− cells (Fig. 7A). Substrates also accumulated in Rce1−/− cells (Fig. 7A). However, no such accumulation was detectable in Zmpste24−/− cells (Fig. 7A).

Similarly, no accumulation of methylatable substrates was detected in extracts of Zmpste24−/− livers, kidneys, or hearts (not shown).

In a separate experiment, we tested the ability of membranes from Zmpste24−/− cells to convert the seven amino-terminal amino acids from P1 (farnesylated factor precursor), generating a shorter intermediate, P2, which can be resolved from P1 by SDS-PAGE (9, 10, 29). We produced a metabolically labeled P1 intermediate in ste24Δ yeast, then tested the ability of Zmpste24−/− and Zmpste24+/+ membranes to cleave the amino terminus from P1, converting it to P2 (Fig. 6). Membranes from Zmpste24+/+ ES cells and fibroblasts converted P1 to P2, and this activity was blocked by 1,10-phenanthroline (Fig. 6A). Membranes from Zmpste24−/− ES cells and fibroblasts lacked this activity (Fig. 6A). Similarly, membranes from the liver, heart, and skeletal muscle of Zmpste24−/− mice converted P1 to P2, whereas membranes from Zmpste24−/− mice did not (Fig. 6B).

We have sought to define the physiologic importance of the two endoproteases and the methyltransferase involved in the "post-isoprenylation" processing of proteins containing a CAAX motif (2). We have shown that ICMT and Rce1 are essential for the processing of the Ras proteins and that both are required for embryonic development. The involvement of Rce1 and ICMT in Ras protein processing was not particularly surprising, given that the corresponding yeast genes, RCE1 and
STE14, had already been shown to be involved in the processing of yeast Ras2p (4, 5, 31, 32). The fact that knockouts of the STE14 gene-targeting vector eliminated the critical HE zinc-binding domain and introduced a frameshift, and we had demonstrated that the yeast membranes contained active Zmpste24 by showing that they promoted the processing and methylation of the 15-mer CAMQ peptide. The use of a CAMQ substrate, as shown in Fig. 3D.

In yeast, the only known substrate for Ste24p is the mating pheromone a-factor (2, 5, 6, 8–10, 29). Although no a-factor orthologue has yet been identified in mammals, zinc metalloproteases similar to Ste24p can be readily identified in many species throughout the plant and animal kingdoms (2, 9). Interestingly, the level of amino acid sequence identity is higher for yeast Ste24p and mouse Zmpste24 than for yeast Rce1p/mouse Rce1 or for yeast Ste14p and mouse ICMT (2). The high level of sequence similarity initially led us to suspect that Zmpste24 might also encode a key housekeeping protein required for mouse survival. This suspicion was not borne out. Zmpste24-deficient mice developed normally and were fertile. The absence of a lethal phenotype cannot be attributed to a leaky phenotype or a poorly designed knockout experiment. Our gene-targeting vector eliminated the critical HE zinc-binding domain and introduced a frameshift, and we had proven with yeast expression studies, even before embarking on the mouse experiments, that the mutation inactivated the enzyme.

In assessing the biochemical phenotype associated with mouse Zmpste24 deficiency, we used yeast a-factor as a surrogate substrate, since no natural mammalian substrates for Zmpste24 have been identified. Nonetheless, our biochemical studies uncovered new and intriguing findings. First, we found that an enzymatic activity capable of cleaving the amino terminus of a-factor is expressed at readily detectable levels in wild-type mouse ES cells and in multiple tissues of wild-type mice. This enzymatic activity was absent in the setting of Zmpste24 deficiency. Thus, Zmpste24 was the only mammalian enzyme capable of processing the amino terminus of a-factor in vitro; no redundant “Ste24p/Zmpste24-like” amino-terminal cleavage activities were detected. We also demonstrated that Zmpste24-deficient cells are deficient in a specific CAAX endoprotease activity, the ability to cleave the a-AAX from a CAMQ a-factor peptide. The use of a CAMQ a-factor peptide was essential for these experiments because it cannot be cleaved by Rce1 (5, 6).

One possible conclusion from the phenotype of the Zmpste24+/– mice is that there are simply no essential Zmpste24 substrates. Alternatively, developmentally essential Zmpste24 substrates may exist but are processed, at least to a degree, by other proteases. In this regard, it is worthwhile pointing out that STE24-deficient yeast are not completely deficient in their ability to produce mature a-factor (2, 5, 6, 9). Some mature a-factor is produced because the carboxyl terminus of a-factor precursor can be cleaved by Rce1p and because the Axl1p-mediated amino-terminal cleavage step proceeds, albeit inefficiently, in the absence of Ste24p. Thus, it is quite conceivable that the viability of Zmpste24+/– mice results from processing of Zmpste24 substrates by other enzymes.

The only known Ste24p substrate in yeast, a-factor, is meth-
lated after the proteolytic release of the carboxyl-terminal tripeptide (4). If unique Zmpste24 CAAX protein substrates were to exist in mammalian cells (i.e., substrates not cleaved by Rce1), we predicted that we would be able to document an accumulation of methylatable substrates in Zmpste24-deficient cells. This prediction was not upheld. No accumulation of methylatable substrates was detected either in Zmpste24−/− ES cells or in a variety of Zmpste24−/− tissues. In contrast, an accumulation of methylatable substrates was easily detectable in both Rce1-deficient cells and ICMT-deficient cells. Recombinant Zmpste24 could not cleave the accumulated substrates in Rce1 knockout cells. How should these studies be interpreted? One could properly conclude that these studies show that a greater number of methylatable protein substrates accumulate in the setting of Rce1 or ICMT deficiency than in Zmpste24 deficiency. One could also conclude that Zmpste24 cannot process the uncleaved substrates in Rce1-deficient cells. However, we would be reluctant to conclude that mammalian cells have minus of yeast as well as the human enzyme faithfully cleave the amino terminus of Ste24p and mouse Zmpste24 is remarkable. Mouse Zmpste24 substrates are cleaved by other enzymes (e.g. Rce1). Unique Zmpste24 substrates may accumulate in Zmpste24 deficiency but at concentrations too low to yield levels of carboxyl methylation above background.

The conservation in the enzymatic specificities for yeast Ste24p and mouse Zmpste24 is remarkable. Mouse Zmpste24 as well as the human enzyme faithfully cleave the amino terminus of yeast a-factor. Moreover, both mouse Zmpste24 and yeast Ste24p share the property of being able to cleave the carboxyl terminus of a wild-type a-factor and a CAMQ a-factor mutant, but not a CTLM a-factor mutant. The striking conservation of Ste24p/Zmpste24 specificities makes it tempting to speculate that an a-factor orthologue is lurking in the mammalian genome. We have not yet identified such a protein, but recognizing an open reading frame as short as 36 amino acids (the length of the a-factor precursor) could be quite challenging. This challenge is compounded when one considers the fact that a-factor-like CAAX proteins from diverse organisms (e.g. Saccharomyces cerevisiae, Schizosaccharomyces pombe, Ustilago maydis, Ustilago hordei, Filobasidiella neoformans) exhibit only limited sequence similarities.

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