

Original Research Article

The Calpain Small Subunit Gene Is Essential: Its Inactivation Results in Embryonic Lethality

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Summary

Creation of transgenic (knockout) mice deficient in calpain small (30 kDa) subunit gene was undertaken to clarify the proposed role of the small subunit for calpain proteolytic activity and to gain insight into the importance of the gene in the whole animal. The gene was targeted and disrupted in embryonic stem cells by homologous recombination, and chimeric mice were generated. Heterozygous F₁ generation mice were crossed to obtain F₂ generation. Among F₂ generation mice, we found only wild-type and heterozygous animals in the 80 pups genotyped to date; no homozygous mice have been found, although 20 were expected. The heterozygotes had no apparent phenotypic abnormalities. Analysis of their tissues revealed no significant difference in mRNA expression, protein content, or proteolytic activity in comparison with their wild-type littermates. Genotyping of fetuses at different stages of development also revealed only wild-type and normal heterozygous fetuses. No moribund embryos or resorption sites were observed in the uterine cavity. The results indicate that at least one normal allele is essential for postnatal survival. Disruption of both alleles appears to be lethal in very early fetal development.

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INTRODUCTION

Calpain, a family of cysteine proteases, can be divided into either ubiquitous or tissue-specific isoforms. μ -Calpain and

m-calpain are ubiquitously present in mammalian tissues and require micromolar and millimolar amounts of calcium, respectively, for in vitro activity. These heterodimers are composed of a distinct catalytic 80-kDa subunit and a common 30-kDa subunit. The catalytic subunit is itself subdivided into four domains (I–IV). Domain I is cleaved at its N terminus during autolytic activation. Domain II contains the essential cysteine and histidine residues of the active site. Domain III has no obvious sequence homology with other proteins, and its function is unknown. Domain IV is a calmodulin-like domain that contains five EF-hand helix-loop-helix Ca²⁺-binding motifs. The small (or regulatory) subunit is subdivided into two domains, V and VI; the first is an N-terminal glycine-rich domain, and the second, a calmodulin-like domain homologous with domain IV of the catalytic subunit. The calmodulin-like domains of two subunits associate to form the heterodimeric calpain. Despite extensive knowledge of their biochemical and molecular properties, the function of calpains in vivo is unknown. They have been implicated in a wide variety of processes, including apoptosis, cell cycle regulation, proliferation, differentiation, cytoskeletal reorganization, secretion, signal transduction, and long-term potentiation (1–4).

More recently, there has been controversy as to whether or not the small subunit is essential or dispensable for calpain activity. Earlier reports indicated that both subunits were required for full expression of proteolytic activity. More recently, however, the catalytic subunit has been shown to have full activity even in the absence of the small subunit (5). On the other hand, the baculovirus-expressed monomeric catalytic subunit shows less activity than the coexpressed heterodimer (6). Furthermore, more 80-kDa protein is found when the small subunit is coexpressed. These findings have led to an alternative proposal that the primary role of small subunit may be to stabilize and

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promote proper folding of the catalytic subunit rather than to direct catalysis.

Investigators also question whether the small subunit remains associated with the catalytic subunit when activated. Because calpain dissociates into subunits in the presence of Ca^{2+} (7, 8), calpain has been proposed to function as a monomer of the 80-kDa subunit *in vivo*. Under this scenario, one would expect the small subunit to play a very minor role in calpain-mediated cellular events. Contrary to this observation, however, both subunits were coprecipitated by a monoclonal antibody specific for the small subunit in the presence of Ca^{2+} concentrations that permit catalytic activity (9). This finding argues for the continued association of both subunits during the proteolysis of substrates and for the activity of both calpain isoforms being affected equally by the small subunit.

To resolve these discrepancies, to address the role of the small subunit in the calpain proteolytic systems, and to broaden our understanding of the physiological roles of calpain, we initiated a program (10) and the importance of the small subunit is being assessed by the effect of its absence in the whole animal.

EXPERIMENTAL PROCEDURES

Isolation of Mouse Calpain Small Subunit Genomic Clones. Mouse strain 129/SvJ bacterial artificial chromosome library was screened by using rat calpain small subunit cDNA. The 550-bp piece of rat calpain small subunit cDNA in pT7-7f21k plasmid (a gift from Dr. John Elce, Queen's University, Canada) was released by *NdeI/PstI* digestion, gel-purified, labeled with ^{32}P by random priming, and used as a hybridization probe (Genome Systems, St. Louis, MI). The positive clone (13931) was digested with *BamHI*, *EcoRI*, and *HindIII* and then subcloned. Two probes, 4F-139R (250 bp) and 162F-320R (1400 bp) were generated by polymerase chain reaction (PCR) with primers designed from the rat calpain 30-kDa subunit cDNA map and using our newly isolated mouse genomic clone 13931 as a template. The probe 4F-139R extended from the base pair at position 4 in exon 4 to bp 139 near the end of exon 5 and included a relatively short intron, according to the human calpain small subunit genomic DNA sequence (11). Probe 162F-302R extended from bp 162 in exon 6 and straddled a long intron after exon 6 and a short intron between exons 7 and 8. These PCR-generated probes were utilized to identify various subcloned genomic DNAs, including H4 and G12.

PCR to Analyze *F1* Mouse Tail DNA for *neo*^R Gene. The neo forward primer (5'-CTGTGCTCGACGTTGTCCTG-3') and reverse primer (5'-GATCCCTCAGAAGAACTCGT-3') were used according to the PCR protocol described in Perkin-Elmer publication. The size of the neo PCR product was ~560 bp.

DNA Gel Blot Analysis. The standard DNA gel blot protocol was used (12). Briefly, DNA (5–10 g) was digested overnight with 200 U of *HindIII* (New England BioLabs., Beverly, MA) in the presence of bovine serum albumin and spermidine. The reaction mixture was electrophoresed on 0.7% agarose gel, denatured, and transferred to nylon membrane (Genescreen Plus;

NEN/Dupont). The prepared membrane was hybridized with the 5'-probe (300 bp, *MfeI/HindIII* digest of subclone H4) or with the 3'-probe (260 bp, *NcoI/HindIII* digest of subclone G12). These DNA fragments were gel-purified, labeled with ^{32}P by using a random primed DNA labeling kit (Amersham, Piscataway, NJ), and used as hybridization probes.

For routine genotyping, a simpler DNA gel blot protocol was adopted: Tail DNA or fetal tissue DNA was digested with *HindIII* and hybridized with the 600-bp *EcoRI* fragment immediately upstream of the 5' homology arm (H4).

RNA Gel Blot Analysis. Total RNA was extracted from mouse tissues and processed for RNA gel blot analysis as described (12). An *XbaI-HindIII* digest of pT7-7f21k was radiolabeled with a random primed DNA labeling kit (Amersham) and used as the hybridization probe. The intensity of the mRNA band was quantified by an Ambis 4000 Radioanalytic Imager and normalized to that of 18S rRNA.

Protein Gel Blot Analysis. Approximately 200 mg of fresh tissue was minced, placed in 3.0 ml of buffer (20 mM MOPS, 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, pH 7.5), and homogenized on ice with two 10-s bursts of a Brinkmann Polytron at 60% maximal speed. Supernatant from this homogenate was obtained by centrifugation at 20 000 g for 30 min at 4 °C. The primary antibody was chicken polyclonal antibody that recognized both 80- and 30-kDa subunits (Parke-Davis, Ann Arbor, MI). Secondary antibody was biotin-labeled rabbit anti-chicken IgY (Accurate Chemical and Scientific Corp., Westbury, NY). Tertiary antibody was anti-rabbit antibody conjugated with streptavidin and alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The blot was treated with 5-bromo-4-chloro-3-indoyl phosphate and stained with nitroblue tetrazolium.

Calpain Activity Assay. Supernatant from the tissue homogenate (50–70 mg/ml, 2 ml) was chromatographed on a Reactive Red-agarose column (0.8 × 2 cm) to separate calpain from calpastatin in a single step (13). The proteolytic activity of the chromatographed fractions was determined by using the fluorogenic peptide Suc-Leu-Tyr-7-amino-4-methylcoumarin (Calbiochem, San Diego CA) as substrate (14). Each reaction mixture contained 100 μl (~25 mg/ml) of sample, 100 μl of substrate (5–50 M) and 700 μl of buffer (20 mM MOPS, 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, pH 7.5). The reaction was started by adding 100 μl of Ca (50 mM), incubated at 37 °C for 60 min, and terminated by adding 100 μl of EGTA (100 mM). Fluorescence from the release of the amino-4-methylcoumarin was measured at 360 nm (excitation) and 430 nm (emission).

RESULTS

Construction of the Knockout Vector and Identification of Recombinant Embryonic Stem (ES) Cells

Screening the bacterial artificial chromosome library (129/SvJ) for mouse calpain small subunit genomic clone by

using rat calpain small subunit cDNA as a hybridization probe located two positive clones. One of the clones (13931) contained a 15.5-kb piece of genomic DNA. Mapping and partially sequencing the restriction enzyme site revealed that 13931 genomic DNA extended from the upstream of exon 1 to downstream of final exon 11, according to the human calpain small subunit genomic sequence (11) (Fig. 1A, top). The clone was further digested with *Bam*HI, *Eco*RI, and *Hind*III and subcloned. Subclones H4 and G12 were identified by PCR-generated probes (4F-139R and 162F-320R) and were found to contain 8.8 and 6.7 kb of *Hind*III-digested DNA, respectively. Partial sequencing and alignment against the human genomic sequence further identified H4 as the DNA that extended from the first through

the seventh exon and showed that G12 extended from exon 7 through exon 11. H4 and G12 were then utilized to construct a knockout vector in plasmid pGTN29. A 4.4-kb *Eco*RI digest of H4 that included exons 1 through 3 and a 3.3-kb *Bam*/Nhe digest of G12 that included exons 9 and 10 were ligated to two ends of a selectable marker neomycin resistance gene (*neo*^R) cassette. The *neo*^R gene thus replaced a 4.3-kb region spanning exons 4 through 8 of the calpain small subunit gene. The total size of the construct was 12.3 kb (Fig. 1A, bottom).

The knockout vector (100 μ g) was linearized at a unique *Not*I site and electroporated into 129/SvJ mouse ES cells (1×10^7 /ml). Transfected cells were then plated onto irradiated mouse embryonic fibroblast feeder layers. The ES cells were subjected to

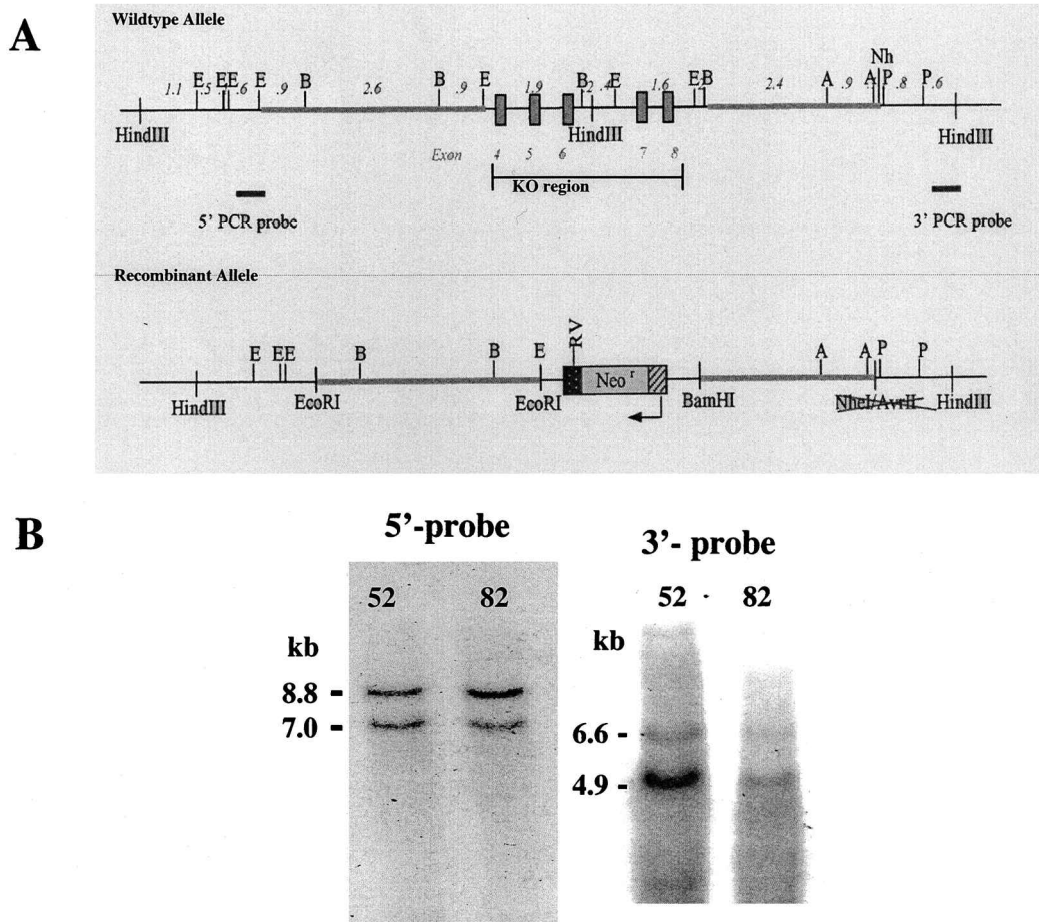


Figure 1. (A) Mouse calpain small subunit genomic clone 13931. (Top) Map of restriction enzyme sites and locations of the targeted exons: The restriction sites were mapped and the exon-intron boundaries were determined by partial sequencing and alignment against rat cDNA and human genomic sequence. The approximate positions of 5'- and 3'-PCR probes (4F-139R and 162F-320R) used to identify H4 and G12 subclones are indicated. (Bottom) Knockout vector construct: A 4.4-kb *Eco*RI digest of H4 and a 3.3-kb *Bam*/Nhe digest of G12 DNA were ligated to the 5'- and 3'-ends, respectively, of a *neo*^R gene cassette to form the knockout vector. E, *Eco*RI; B, *Bam*HI; A, *Avr*II; P, *Pst*I; RV = *Eco*RV. (B) DNA gel blot analysis of homologously recombinant ES cells. Left panel: *Eco*RV/*Hind*III digest of genomic DNA from clones 52 or 82, hybridized with the 5' probe (300 bp, *Mfe*I/*Hind*III digest of H4). The expected 8.8-kb band from the wild-type allele and the 7.0-kb band from the recombinant allele are shown. Right panel: *Eco*RI/*Hind*III digest of genomic DNA from clones 52 or 82, hybridized with the 3' probe (260 bp, *Nco*I/*Hind*III digest of G12). The expected 4.9-kb band from the wild-type allele and the 6.6-kb band from the recombinant allele are shown.

antibiotic selection with geneticin (300 $\mu\text{g/ml}$). After 7–8 days, the 192 (8%) colonies that survived were analyzed for homologous recombination by DNA gel blot analysis. Two probes were utilized: a 5' probe (300 bp, *MfeI/HindIII* digest of H4) and a 3' probe (260 bp, *NcoI/HindIII* digest of G12). These probes were chosen so that *EcoRV/HindIII* digestion of the ES cell genomic DNA and hybridization with the 5' probe would yield a 8.8-kb band from the wild-type allele and a 7.0-kb band from the recombinant allele, whereas *EcoRI/HindIII* digestion of ES cell genomic DNA and hybridization with the 3' probe should yield a 4.9-kb band from the wild-type and a 6.6-kb band from the recombinant allele. Four ES cell colonies had the predicted banding pattern. The banding patterns of two of the colonies (nos. 52 and 82) are shown in Fig. 1B.

Chimeric Mice and F₁ and F₂ Generation Mice

Recombinant ES clones no. 52 and no. 82 were expanded, and each was separately injected into C57BL/6 mouse blastocysts. The blastocysts were implanted into recipient pseudopregnant female mice. Three and four male chimera were generated from the no. 52 and no. 82 clones, respectively. All of the chimera were $\geq 90\%$ agouti. Chimera were backcrossed to C57BL/6 mice to generate F₁ wild-type (+/+) and heterozygous (+/-) mice. Initially, we screened tail DNA of the 3-week-old F₁ mice by PCR for the presence of the *neo^R* gene. Tissues from *neo^R*-positive F₁ mice were further analyzed by DNA gel blot. The results of genotyping by the two methodologies were always in agreement. Three pairs of F₁ (+/-) male and female mice were intercrossed to produce F₂ generation litters. Two sets of litters from each pair of parents were genotyped by DNA gel blot analysis with three different probes, the 5' probe (300 bp, *MfeI/HindIII* digest of H4), the 3' probe (260 bp, *NcoI/HindIII* digest of G12), and the simpler 5' probe (600 bp, *EcoRI* digest of H4). The *HindIII* digest of tail DNA and hybridization with the simpler 5' probe should yield a 8.8-kb band from wild-type and a 13.0-kb band from the recombinant offspring (Fig. 2). Genotyping the 60 F₂ pups pooled from the seven litters originating from clone 52 showed 12 wild-type and 48 heterozygous but no homozygous mice. Similarly, of the 20 F₂ pups from the two litters originating from an independent clone (no. 82), 6 were wild-type and 14 were heterozygous mice, but none was homozygous (Fig. 2, top). Thus, of the total 80 F₂ progeny genotyped to date, the observed ratios of wild-type:heterozygous:homozygous offspring were 1:3.4:0, a value significantly different from the expected Mendelian ratios of 1:2:1. These results indicate that at least one normal allele is essential for postnatal survival.

Timed matings were carried out to obtain fetuses at differing stages of development, delivered abdominally at the 9th, 12th, and 16th days of gestation. At the earliest day examined, only the wild-type and heterozygous fetuses were found in 1:2 ratios (Fig. 2, bottom). Heterozygous fetuses exhibited no developmental abnormalities. Neither a moribund fetus nor embryo resorption sites were found in the uterine cavity, suggesting that embryonic lethality occurred earlier than day 9.

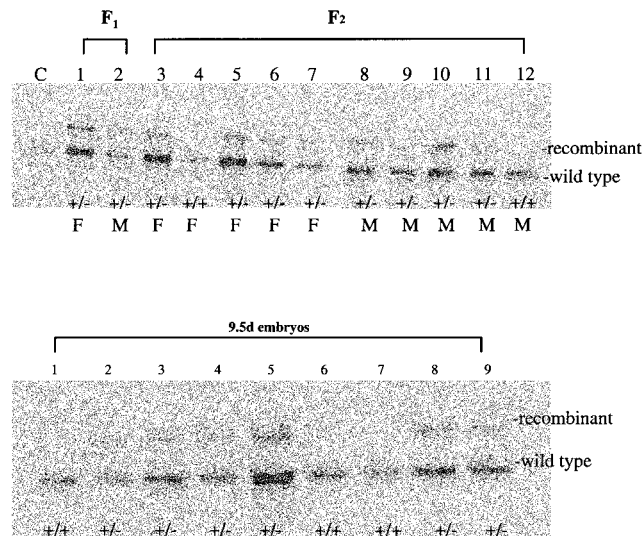


Figure 2. Genotypes of F₁ parents, F₂ pups, and 9-day-old embryos. (Top) A representative DNA gel blot analysis of *HindIII*-digested tail DNA (5–10 μg) from F₁ parents and from one representative set of their litter (10 pups), hybridized with the 5' probe (600 bp, *EcoRI* digest of H4). Only the 8.8-kb band is present in the wild-type (+/+) mice, whereas an additional 13.0-kb band is present in the heterozygous (+/-) mice. Distribution of genotype (2 wild types and 8 heterozygotes) and sex (5 females and 5 males) of the F₂ pups is indicated. (Bottom) A representative gel blot analysis of DNA preparations from the yolk sac or whole body of 9-gestation-day-old F₂ embryos. The wild-type embryos have a single 8.8-kb band, and the heterozygous embryos have an additional 13-kb band.

Phenotypes of Heterozygous F₂ Mice

Male and female heterozygous mice were present in equal frequency, were healthy and fertile, and had no overt physiological disadvantage in appearance or somatic growth at maturity (body weight, behavior, or organ size) compared with their wild-type littermates. The amounts of small subunit mRNA in selected organs and tissues (brain, lung, liver, and skeletal muscle) were determined by RNA gel blot with rat cDNA as the hybridization probe. Although the amount of mRNA expression varied in different tissues, in agreement with the previous data (15), there was no substantial decrease in the signal from a given tissue of a heterozygous mouse relative to the wild-type animal ($n = 3$) (Fig. 3, top). Protein gel blot analysis of calpain protein was determined by using polyclonal antibody that recognized both subunits. The protein bands present in heterozygotes showed similar amounts as those of the wild-type mice (Fig. 3, middle). Assaying proteolytic activities by using fluorogenic synthetic substrate showed no significant differences between the heterozygous and wild-type tissues ($n = 3$) (Fig. 3, bottom).

DISCUSSION

The small subunit has always been present in μ and m-calpain isolated from natural sources. Hence, it has long been assumed

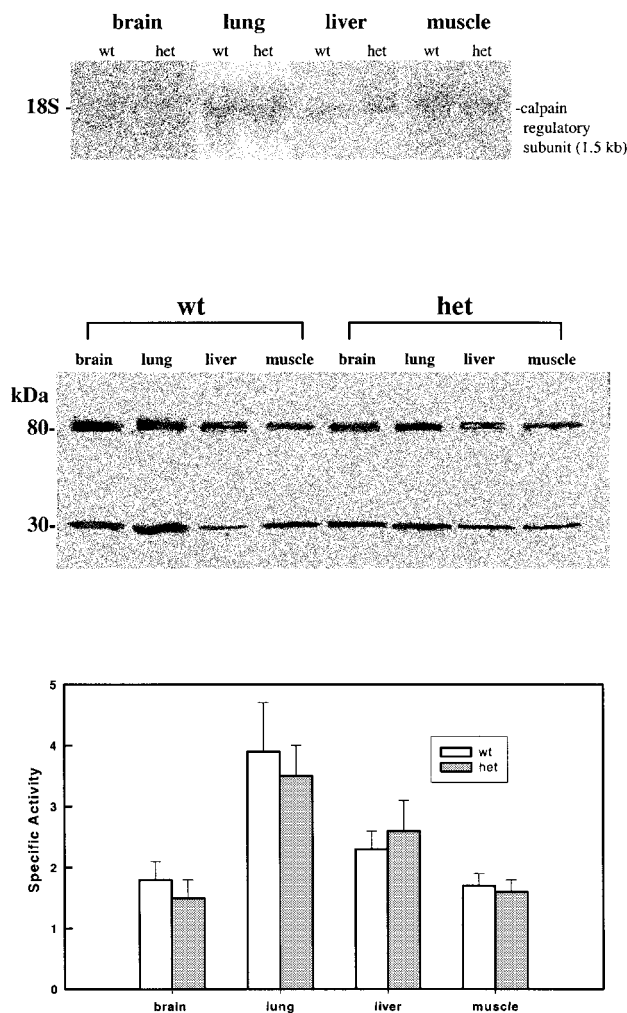


Figure 3. Phenotypes of F_2 heterozygous mice. (Top) Gel blot analysis of total RNA prepared from brain, lung, liver, and skeletal muscle of wild-type (wt) and heterozygous (het) mice. Ten micrograms of each RNA was analyzed by using the *Xba*I/*Hind*III digest of plasmid pT7-7f21k as a hybridization probe. In each tissue, a 1.5-kb band of small subunit mRNA was detected just below the 18S rRNA mark. (Middle) Protein gel blot analysis. Calpain 80-kDa catalytic and 30-kDa small subunit protein bands were detected by using chicken polyclonal antibody to recombinant m-calpain. Biotin-labeled rabbit anti-chicken antibody was the secondary antibody, and the tertiary antibody was conjugated with streptavidin and alkaline phosphatase. (Bottom) Calpain activity in various tissues. Calcium-dependent proteolytic activity of the Reactive Red-agarose chromatographic fraction was measured by the increased fluorescence resulting from the release of 7-amino-4-methylcoumain from the peptide substrate.

to be involved in regulation of proteolytic activity. Although each subunit is transcribed by a unique, single-copy gene, the 5'-upstream regions of both subunits show 50% homology and share features with housekeeping genes, including the lack of a

TATA box and a very high GC content. The Sp1 and AP-1 recognition sites are also present in both, suggesting that expressions of these two genes are tightly coupled and coregulated at the transcriptional level (2, 14). The gene structure of the human small subunit indicates that although the exon 1 and the start of exon 2 are untranslated, exons 2 and 3 encode the N-terminal glycine-rich domain of the subunit. Of the five EF-hand calcium-binding motives that follows, EF-1 is encoded by exons 4 and 5, EF-2 by exons 6 and 7, and EF-3, EF-4, and EF-5 are each encoded by the single exons 8, 9, and 10, respectively. On the basis of this scheme, our knockout vector is such that exons 4-8 were replaced by a neo^R cassette, thus eliminating calcium-binding domains EF-1, -2, and -3. The locus mutated by this vector, however, was not expected to encode a transcript that would be translated into a functional protein.

In the present study, a homologous recombination of the knockout vector with the calpain small subunit gene generated only healthy heterozygous and wild-type mice. Even at the earliest gestation day (9.5) examined, no homozygous fetus or embryo resorption sites were found. These results strongly suggest that the null mutation of the small subunit gene resulted in early embryonic lethality. In recent years, it has become apparent that controlled proteolysis of the growth-regulating proteins is an essential feature of normal cell division and growth. Indeed, previous studies with calpain inhibitors suggested that calpain was involved in regulating cell proliferation (16-18). More recently, calpain inhibitors were found to block cell progression into the S-phase of serum-stimulated fibroblasts (19). Tumor suppressor p53, known to control the restriction point R in the G₁ phase, is cleaved by calpain (20). Cyclin D1, necessary for G₁ to S-phase transition, is also proteolyzed by calpain (21). Moreover, during the various phases of mitosis, calpain relocates in A431 (22) and PtK1 cells (23). In the latter, microinjection of m-calpain promotes the onset of mitosis and accelerates the metaphase to anaphase transition. During meiosis in *Xenopus* eggs (24) and vertebrate eggs (25), the cleavage of c-mos by calpain leads to dephosphorylation of cyclin, and the subsequent cyclin cleavage permits meiosis to proceed from metaphase through anaphase. In addition, a highly ordered, irreversible reorganization of cellular contents involving limited proteolysis of cytoskeletal elements has also been shown to occur during mitosis. Indeed, in *Drosophila*, immunohistochemical analysis depicts concerted movement of calpain in the dynamic rearrangement of the actin-related cytoskeleton immediately after fertilization (26). More significantly, depletion of the calpain regulatory subunit using antisense oligodeoxyribonucleotide inhibited cell proliferation (27).

The results of those cellular studies provide important clues and reasons for the absence of viable homozygous mice in our present endeavor. Thus, inhibition of the calpain catalytic subunit in the studies led to aborted cell division, growth, and proliferation. Deletion of the small subunit gene in our present study led to embryonic lethality. Both approaches reached the same endpoint. This convergence could arise if the small subunit is an essential and integral part of calpain function.

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REFERENCES

- Carafoli, E., and Molinari, M. (1998) Calpain: a protease in search of a function? *Biochem. Biophys. Res. Commun.* **247**, 193–203.
- Suzuki, K. (1990) The structure of calpains and the calpain gene. In *Intracellular Ca²⁺-Dependent Proteolysis* (Mellgren, R. L., and Murachi, T., eds.) pp. 25–35, CRC Press, Boca Raton, FL.
- Suzuki, K., Sorimachi, H., Yoshizawa, T., Kinbara, K., and Ishiura, S. (1995) Calpain: novel family members, activation, and physiological function. *Biol. Chem. Hoppe-Seyler* **376**, 523–529.
- Wang, K. K. W., and Yuen, P.-W. (1994) Calpain inhibition: an overview of its therapeutic potential. *Trends Pharmacol. Sci.* **15**, 412–419.
- Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S., and Suzuki, K. (1995) A catalytic subunit of calpain possesses full proteolytic activity. *FEBS Lett.* **358**, 101–103.
- Graham-Siegenthaler, K., Gauthier, S., Davies, P. L., and Elce, J. S. (1994) Active recombinant rat calpain II. Bacterially produced large and small subunits associate both *in vivo* and *in vitro*. *J. Biol. Chem.* **269**, 30457–30460.
- Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S., and Suzuki, K. (1995) Calpain dissociates into subunits in the presence of calcium ions. *Biochem. Biophys. Res. Commun.* **208**, 376–383.
- Suo, S., Koike, H., Sorimachi, H., Ishiura, S., and Suzuki, K. (1999) Association and dissociation of the calcium-binding domains of calpain by Ca²⁺. *Biochem. Biophys. Res. Commun.* **257**, 63–66.
- Mellgren, R. L., and Lane, R. D. (1988) Myocardial calpain 2 is inhibited by monoclonal antibodies specific for the small noncatalytic subunit. *Biochim. Biophys. Acta* **954**, 154–160.
- Bronson, S. K., and Smithies, O. (1994) Altering mice by homologous recombination using embryonic stem cells. *J. Biol. Chem.* **269**, 27155–27158.
- Miyake, S., Emori, Y., and Suzuki, K. (1986) Gene organization of the small subunit of human calcium-activated neutral protease. *Nucleic Acids Res.* **14**, 8805–8817.
- Maniatis, T., Fritsch, E. F., and Sambrook, W. (1982) *Molecular Cloning: A Laboratory Manual*, John Wiley & Sons, Inc., Cold Spring Harbor, NY.
- Clark, A. F., DeMartino, G. N., and Croall, D. E. (1986) Fractionation and quantification of calcium-dependent proteinase activity from small tissue samples. *Biochem. J.* **235**, 279–282.
- Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* **259**, 12489–12494.
- Emori, Y., Kawasaki, H., Imajoh, S., Kawashima, S., and Suzuki, K. (1986) Isolation and sequence analysis of cDNA clones for the small subunit of rabbit calcium-dependent protease. *J. Biol. Chem.* **261**, 9472–9476.
- March, K. L., Wilensky, R. L., Roeske, R. W., and Hathaway, D. R. (1993) Effect of thiol protease inhibitors on cell cycle and proliferation of vascular smooth muscle cells in culture. *Circ. Res.* **72**, 413–423.
- Mellgren, R. L., Shaw, E., and Mericle, M. T. (1994) Inhibition of growth of human TE2 and C-33A cells by the cell permeant calpain inhibitor benzyloxycarbonyl-leu-leu-tyr r diazomethyl ketone. *Exp. Cell. Res.* **215**, 164–171.
- Mellgren, R. L., Lu, Q., Zhang, W., Lakkis, M., Shaw, E., and Mericle, M. T. (1996) Isolation of a Chinese hamster ovary cell clone possessing decreased μ -calpain content and a reduced proliferative growth rate. *J. Biol. Chem.* **271**, 15568–15574.
- Zhang, W., Lu, Q., Xie, Z.-J., and Mellgren, R. L. (1997) Inhibition of the growth of WI-38 fibroblasts by benzyloxycarbonyl-Leu-Leu-Tyr r diazomethyl ketone: evidence that cleavage of p53 by a calpain-like protease is necessary for G₁ to S-phase transition. *Oncogene* **14**, 255–263.
- Kubbutat, M. H. G., and Vousden, K. H. (1997) Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol. Cell. Biol.* **17**, 460–468.
- Choi, Y. H., Lee, S. J., Nguyen, P. M., Jang, J. S., Lee, J., Wu, M.-L., Takano, E., Maki, M., Henkart, P. A., and Trepel, J. B. (1997) Regulation of cyclin D1 by calpain protease. *J. Biol. Chem.* **272**, 28479–28484.
- Shoji-Kasai, Y., Senshu, M., Iwashita, S., and Imahori, K. (1988) Thiol protease-specific inhibitor E-64 arrests human epidermoid carcinoma A431 cells at mitotic metaphase. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 146–150.
- Schollmeyer, J. E. (1988) Calpain II involvement in mitosis. *Science* **240**, 911–913.
- Watanabe, N., Van de Woude, G. F., Ikawa, Y., and Sagata, N. (1989) Specific proteolysis of the *c-mos* protooncogene product by calpain on fertilization of *Xenopus* eggs. *Nature* **342**, 501–511.
- Sagata, N., Watanabe, N., Van de Woude, G. F., and Ikawa, Y. (1989) The *c-mos* proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* **342**, 512–518.
- Emori, Y., and Saigo, K. (1994) Calpain localization changes in coordination with actin-related cytoskeletal changes during early embryonic development of *Drosophila*. *J. Biol. Chem.* **269**, 25137–25142.
- Zhang, W., Lane, R. D., and Mellgren, R. L. (1996) The major calpain isozymes are long-lived proteins. *J. Biol. Chem.* **271**, 18825–18830.