Ubiquitously expressed μ- and m-calpain proteases are implicated in development and apoptosis. They consist of 80-kDa catalytic subunits encoded by the capn1 and capn2 genes, respectively, and a common 28-kDa regulatory subunit encoded by the capn4 gene. The regulatory subunit is required to maintain the stability and activity of μ- and m-calpains. Accordingly, genetic disruption of capn4 in the mouse eliminated both ubiquitous calpain activities. In embryonic fibroblasts derived from these mice, calpain deficiency correlated with resistance to endoplasmic reticulum (ER) stress-induced apoptosis, and this was directly related to a calpain requirement for activation of both caspase-12 and the ASK1-JNK cascade. This study provides compelling genetic evidence for calpain’s role in caspase-12 activation at the ER, and reveals a novel role for the ubiquitous calpains in ER-stress induced apoptosis and JNK activation.

Calpains are a family of Ca2+-dependent intracellular cysteine proteases. By cleaving their protein substrates, ubiquitously expressed μ-calpain and m-calpain are implicated in a wide variety of biological functions including cell migration, cell cycle regulation, differentiation, and apoptosis (reviewed in Ref. 1). Both μ- and m-calpains are heterodimers, consisting of a distinct large 80-kDa catalytic subunit, encoded by the genes capn1 and capn2, respectively, and a common small 28-kDa regulatory subunit encoded by the capn4 gene. The small subunit is essential to calpain activities, as shown by in vitro biochemical studies where a 25-amino acid truncation at the C terminus abolished all detectable calpain activity (2). This provided the rationale for the first reported capn4 knock-out mouse model, which interrupted the coding sequences in exon 9 (3, 4) and was predicted to truncate 38 C-terminal amino acids from the small subunit. The resulting hypothetical small subunit was not detectable, the steady-state levels of μ-80, m-80 catalytic subunits were reduced, and no calpain activity was observed (4).

More recently, we have developed a conditionally targeted capn4 locus by insertingloxP sites into intron 8 and the noncoding region of exon 11. This allows conditional knock-out of capn4 by Cre-mediated recombination. Although a hypothetical small subunit protein with a 60-amino acid deletion at the C terminus might still be produced, no detectable small subunit was detected, probably because of destabilization of the hypothetical truncated protein. Expression of the large subunit was also greatly diminished, supporting the proposed role for the small subunit in stabilizing large subunits. As expected, mouse embryonic fibroblasts (MEFs) from these knockouts also lacked any detectable ubiquitous calpain activity.2 Many reports on calpain function are based on using small molecule inhibitors, which lack specificity. In contrast, this genetic knock-out model is completely selective, and therefore provides a powerful tool to address the physiological functions of the ubiquitous calpains.

Ubiquitous μ- and m-calpains have been suggested to participate in apoptosis by cleaving either pro-apoptotic or anti-apoptotic proteins like p53, Bcl-2, and Bax, depending on the nature of the stimuli and type of cells involved (6–8). Among these studies, m-calpain was proposed to play a role in a distinct apoptotic pathway initiated by ER stress (9). The ER plays key roles in protein biosynthesis, modification, folding, and trafficking, and it is also the major pool for calcium storage. Perturbation of ER homeostasis abolishes protein folding, and the consequent accumulation of unfolded proteins in the ER imposes a so-called ER stress on the cell. The ER membrane residing proteins IRE1, Perk, and ATF6 are ER stress sensors, which are maintained in an inactive monomeric state by binding to the chaperone protein Bip. During ER stress, the release of Bip activates these sensors to convey their protective responses, known as the unfolded protein response (UPR). This includes enhancing the transcription of genes encoding ER chaperones and the attenuation of general protein synthesis (10). Autophosphorylated IRE1 possesses an endoribonuclease activity that alternatively splices the mRNA encoding the transcription factor XBP-1, thus turning on chaperone genes (11). The activated Perk kinase phosphorylates eIF-2α and inhibits general protein synthesis (12). Also, the unfolded proteins trigger ATF6 cleavage by the Site-1 and Site-2 proteases to release its cytoplasmic domain, which then enters the nucleus and activates Bip transcription (13).

ER stress can be induced by pharmacological agents such as the calcium ionophore A23187, the Ca2+ pump inhibitor thapsigargin, the N-linked glycosylation inhibitor tunicamycin, the ER to Golgi transport inhibitor brefeldin A, and inhibitors of disulfide bond formation such as dithiothreitol (14). Excessive ER stress can trigger cellular apoptosis through the activation of caspase-12, which resides on the outside of ER membrane, and is cleaved and activated during ER stress (15). As an initiator caspase, caspase-12 triggers the activation of caspases-9, -7, and -3 in a cytochrome c and Apaf-1-independent manner (16, 17). Caspase-12 knock-out cells are resistant to ER stress-induced apoptosis (15). In vitro biochemical and calpain inhibitor studies in glial cells sug-

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8 This work was supported by an operating grant from the Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

9 The on-line version of this article (available at http://www.jbc.org) contains supplemental data.

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2 Y. Tan, N. Dourdin, C. Wu, T. De Veyra, J. S. Elce, and P. A. Greer, submitted publication.

3 The abbreviations used are: MEF, mouse embryonic fibroblast; ER, endoplasmic reticulum; TG, thapsigargin; TN, tunicamycin; MAP kinase, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ASK1, apoptosis signal-regulating kinase 1; ER, embryonic day; CMV, cytomegalovirus; Cre, Cre recombinase; 7-AAD, 7-aminoactinomycin D; PE, phycoerythrin; AO, acridine orange; IRES, internal ribosome entry site; PARP, polyADP-ribose polymerase; PIPES, 1,4-piperazinediethanesulfonic acid; GFP, green fluorescent protein; TNF, tumor necrosis factor.
gested that m-calpain is responsible for the cleavage of caspase-12 during ER stress (9).

Besides its role in ER stress induced apoptosis, calpain might also participate in a mitochondrial-dependent apoptotic pathway by cleaving and thus releasing apoptosis-inducing factor (AIF) from the inner mitochondrial membrane (18–20). Methamphetamine induced apoptosis of striatal glutamic acid decarboxylase containing neurons through the rapid and simultaneous activation of calpain and caspase-12, and release of AIF and cytochrome c from the mitochondria (18). Calpain activation was persistent under those conditions, so it might have further participated in other downstream apoptotic signaling events. Surprisingly, the activation of caspase-9 in this system was not synergistically accelerated, but delayed (18). In vitro studies suggested that calpain-mediated cleavage of an N-terminal AIF domain is required to allow its release from Bid-permeabilized mitochondria (19). Calpains might also mediate cross-talk between mitochondrial and ER apoptotic pathways depending on the nature of the apoptotic stimuli and the cell type. In mouse pancreatic β-cell islet cells, apoptosis induced by type 2 ryanodine receptor-mediated calcium overloading of mitochondria relies on calpain-10 up-regulation and activation (21). Interestingly, polymorphisms in calpain-10 are linked to human susceptibility to type II diabetes.

The c-Jun N-terminal kinase (JNK) pathway is also activated in response to ER stress by IRE1, a serine/threonine kinase, which gets autophosphorylated during UPR. Activated IRE1 recruits TNF receptor-associated factor 2 (TRAF2), which in turn activates the apoptosis signal-regulating kinase ASK1, which in turn activates the MKK4/JNK cascade (22–24). Although the precise role of JNK in ER stress is not defined, prolonged JNK activation has pro-apoptotic functions downstream of various cell death-inducing stimuli, including TNFα, H2O2, and DNA-damaging agents (25, 26). Interestingly, protein-tyrosine phosphatase 1B (PTP1B) knock-out cells, which are not capable of activating the IRE1-JNK pathway after ER stress are resistant to apoptosis (27).

In this study, we investigated the role of the ubiquitous calpains in ER stress. MEFs from conditional capn4/flxed knock-out animals were used to establish a role for calpain in two key ER stress apoptosis pathways. We show that calpain-deficient MEFs were resistant to thapsigargin (TG) and tunicamycin (TN)-induced apoptosis. This resistance correlated with a defect in the activation of the caspase-12, -9, -3 cascade and the ASK1/MKK4/JNK/c-Jun cascade. These observations provide compelling genetic and biochemical evidence for a central role for calpain in the conversion of Ca2+ signals from the stressed ER to the caspase-12-apoptotic pathway and JNK activation.

**MATERIALS AND METHODS**

Reagents—Tunicamycin, thapsigargin, propidium iodide (PI), trypan blue and acridine orange (AO) were purchased from Sigma. ER-Tracker Red was from Invitrogen Molecular Probes and Annexin-V-PE, 7-aminoactinomycin (7-AAD) was from BD Biosciences/Clontech. Anti-m-calpain, cytochrome-c, Bcl-2, Bax, Bclx, and Bcl2 antibodies were from Santa Cruz Biotechnology. Antibody against both the large (m-80) and small (28-kDa) subunits of m-calpain was previously described (4). The pEGFP-m80 plasmid was previously described (28).

Conditional capn4-targeted Allele—The generation of mice harboring a targeted floxed (loxP-flanked) capn4 allele is described elsewhere.2 Briefly, calpain small subunit coding sequences in exons 9, 10, and 11 were flanked by two loxP sites. An internal ribosome entry site entry (IRES)-lacZ reporter and PGK promoter-Neo selectable gene cassette were inserted after the coding sequences in exon 11 and before the second loxP site to permit X-gal staining and G418 selection. Cre recombinase-mediated excision of sequences between the loxP sites deleted the intervening sequences as well as the lacZ/Neo cassette. We refer to this allele as capn4flxed before Cre-mediated excision, and capn4flxed after excision. In order to achieve ubiquitous loss of calpain activity, these mice were crossed with a transgenic line which expresses Cre recombinase under the control of the cytomegalovirus (CMV) promoter, referred to as cmv-cre (29). The capn4flxed; cmv-cre embryos derived from the capn4flxed; cmv-cre x capn4flxed breeding pairs died at approximately embryonic day (E) 11.5.

**Cell Culture**—Primary fibroblast cultures were established from E10.5 embryos. The yolk sacs, heads, and internal organs were isolated and used for genotyping by Southern blot hybridization. Carcasses were treated with 0.25% collagenase in 20% fetal bovine serum in phosphate-buffered saline for 30 min at 37 °C. Cells were then washed twice with media and plated in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Primary MEFs were used in experiments before the third passage. Spontaneously immortalized MEFs were established as previously described (30). Briefly, primary MEFs were seeded in 60-mm plates at a density of 3 × 105 cells/plate and maintained at that density by passage every 3 days. Immortalized MEFs were established between the 13th and 16th passages.

Lentivirus-mediated Gene Transduction—Lentiviral vectors (pWPXLD, pWPl) were kindly provided by Dr. Trono at University of Geneva. The IRES sequence was excised from pWPl and inserted before the EGFP sequence in pWPXLD using blunt-end ligation. The murine capn4 cDNA was cloned using RT-PCR (forward primer ATCCGACTACGGCTGCATT; reverse primer TTGGGCTGCTGGTGTAG) and inserted at a PmeI site upstream of the IRES. Lentivirus was produced by transfecting 293T cells with pWPXLD and the packaging plasmids pMD2G and pCMV-dR8.74 as described (31). The multiplicity of infection (MOI) was determined by infecting MEFs with different titrations of virus-containing supernatant and measuring the percentage of GFP-positive cells using flow cytometry. To rescue calpain activity, immortalized capn4flxed; cmv MEFs were transduced with lentiviruses encoding recombinant mouse capn4.

**Apoptosis Analysis**—MEFs were seeded at 3 × 106 cells per 60 mm plate and allowed to adhere overnight. On the following day, cells were treated with 3 μg/ml tunicamycin (TN) or 5 μM thapsigargin (TG) for the indicated periods. Mock treated cells were exposed to equivalent concentrations of the vehicle, Me2SO. Apoptosis was measured by 0.4% trypan blue exclusion assays and flow cytometry analysis of PE-conjugated Annexin-V and 7-AAD staining, according to the suppliers instructions. Alternatively, apoptotic cells were quantified by measuring the fraction of cells with a sub-G1 content of PI-staining DNA (32).

**Analysis of Lyosomal Stability**—Cells were exposed to 5 μg/ml acridine orange (AO) for 15 min under standard culture conditions, then released by trypsin treatment and washed twice with medium. Red fluorescence was measured immediately by flow cytometry. Cells with decreased red fluorescence (pale cells) were gated, and their percentages were determined.

**Western Blotting**—Cell monolayers were washed twice with cold phosphate-buffered saline, then incubated for 15 min on ice in lysis buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin). Cell lysates were scraped from the dishes, transferred to Eppendorf tubes, and centrifuged at 13,000 rpm for 20 min to remove insoluble material. Protein content was determined using the Bradford method. Samples (20 μg) were separated by SDS-
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FIGURE 1. Deletion of the calpain small subunit abolishes ubiquitous calpain activity. A, calpain activity was analyzed in vitro using casein zymograms. Lysates from the spontaneously immortalized capn4+/P; cre MEFs (lane 1), capn4+/P; cre MEFs (lane 2), or capn4−/P; cre MEF rescued by lentivirus-mediated transduction of mouse capn4 gene (lane 3) were resolved on casein-containing non-denaturing polyacrylamide gels, and calcium-dependent proteolysis was subsequently performed. B, protein levels of μ-calpain large subunit (μ-80), m-calpain large subunit (m-80), and calpain small subunit were analyzed by Western blotting. Experiments were performed independently at least twice.

PAGE and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using specific primary antibodies, and detected using horseradish peroxidase-coupled secondary antibodies.

Release of Cytochrome c—2 × 10⁶ cells were harvested and washed twice in ice-cold phosphate-buffered saline. The cells were then spun at 200 × g for 5 min. The cell pellet was suspended in 600-μl extraction buffer containing 220 mM mannitol, 250 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM EGTA, 2 mM MgCl2, 1 mM dithiothreitol, and protease inhibitors. After 30 min of incubation on ice, cells were treated with a Dounce homogenizer for 80 strokes and spun for 60 min at 14,000 × g. Supernatants were analyzed for cytochrome c release by Western blotting (33).

In Vitro Analysis of μ- and m-calpain Activities—Calpain activities in cell extracts were detected by casein zymography in a standard Tris-glycine system (34). 40 μg of protein was resolved on 8% non-denaturing polyacrylamide gels containing 1.5 mg/ml casein. Calpain was activated by incubating the gels overnight with 5 mM Ca²⁺, and its activity was assessed by visualization of the casein-cleared regions of the gel after staining with Coomassie Brilliant Blue.

Confocal Fluorescence Microscopy Analysis—To examine m-calpain subcellular localization, NIH3T3 cells were transfected with an m-80-EGFP fusion protein-encoding plasmid (pEGFP-m-80) using Lipofectamine 2000 (Invitrogen). After TG or TN treatment, cells were stained with ER-Tracker Red and imaged by confocal fluorescence microscopy using the green and red channels to visualize m-80-EGFP (35). In primary MEFs, inhibition of N-linked glycosylation in the ER with tunicamycin (TN), or inhibition of the ER-localized Ca²⁺-ATPase with thapsigargin (TG) caused an elevation in cytoplasmic Ca²⁺ levels by about 20%; but no difference was observed between capn4−/−;cre and capn4+/+;cre MEFs (data not shown). To track calpain localization during TG/TN-triggered ER stress, we performed confocal fluorescence microscopy analysis of NIH3T3 fibroblasts transfected with an m-80-EGFP fusion protein-encoding plasmid (28). Recombinant m-80-EGFP was widely dispersed in the cells, and it might undergo further concentration at membranous structures and in the nucleus in response to specific signals such as calcium (35). Calpain has been localized at the plasma membrane, with cytosolic membrane surfaces, and within the lumens of the ER and Golgi apparatus (36).

RESULTS

Capn4+/+ Cells Lack Calpain Activity and Have Reduced Expression of Calpain Subunit Proteins—Spontaneously immortalized MEFs were established from capn4+/+; cre and capn4+/+; cre embryos, and calpain activity was analyzed by in vitro casein zymograms (Fig. 1A). As expected, μ- and m-calpain activity was detected in capn4+/+; cre MEFs, but not in capn4−/−; cre cells. However, when the capn4+/+; cre MEFs were transduced with a lentivirus encoding the mouse capn4 cDNA, both μ- and m-calpain activity were restored. Western blotting analysis of these same cell lysates revealed the expected elimination of small subunit expression in capn4+/+; cre MEFs, but we also noted that steady-state levels of both μ-80 and m-80 were dramatically reduced; and this defect was also rescued by transduction with the capn4 lentivirus (Fig. 1B).

ER Stress Correlated with Calpain ER Translocation and Activation—Calpain inhibitors have been reported to inhibit ER stress-induced apoptosis by inhibiting caspase-12 activation (9). Calpain is widely distributed in the cell cytosol and is associated with membranes; and it might undergo further concentration at membranous structures and in the nucleus in response to specific signals such as calcium (35). Calpain has been localized at the plasma membrane, with cytosolic membrane surfaces, and within the lumens of the ER and Golgi apparatus (36). In primary MEFs, inhibition of N-linked glycosylation in the ER with tunicamycin (TN), or inhibition of the ER-localized Ca²⁺-ATPase with thapsigargin (TG) caused an elevation in cytoplasmic Ca²⁺ levels by about 20%; but no difference was observed between capn4−/−; cre and capn4+/+; cre MEFs (data not shown). To track calpain localization during TG/TN-triggered ER stress, we performed confocal fluorescence microscopy analysis of NIH3T3 fibroblasts transfected with an m-80-EGFP fusion protein-encoding plasmid (28). Recombinant m-80-EGFP was widely dispersed in the cells, however, upon TN or TG treatment, we observed a statistically significant increase in calpain ER localization as assessed by increased spectral overlap of m-80-EGFP and ER-Tracker Red (Fig. 2). This indicated that calpain was recruited to the ER in conjunction with calcium release (Fig. 2B).
Calpain large subunits have a half-life of 5 days, but they are quickly degraded after activation (1). We therefore assessed the stability of calpain large subunits after TG or TN treatment as an indication of their degree of activation. In primary capn4<sup>P/P</sup>; cre MEFs, m-80 subunit levels declined during TG or TN treatment (Fig. 3). Although there was substantially less m-80 subunit in primary capn4<sup>P/P</sup>; cre MEFs, that amount was further diminished after TM or TG treatment (Fig. 3). The μ-80 subunit was not as easily detected in capn4<sup>P/P</sup>; cre MEFs; however, in capn4<sup>+/+</sup>; cre MEFs, we determined that the μ-80 subunit also diminished during TN or TG treatment (Fig. 3). This disappearance of calpain catalytic subunits is consistent with the hypothesis that they are activated and subsequently degraded during ER stress.

Calpain-deficient MEFs Are Resistant to Thapsigargin-induced Cell Death—To establish whether calpain is involved in the ER stress pathway, we compared TN or TG-induced apoptosis in MEFs with or without calpain. We first used primary MEFs (P-MEFs) from capn4<sup>P/P</sup>; cre MEFs were treated with (A) 5 μM TG, or (B) 3 μg/ml TN for the indicated times. μ- and m-calpain large subunits levels in cell lysates were assessed by Western blotting.
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and capn4<sup>P/P</sup>; cre sibling embryos to perform these apoptosis studies. Similar results were then obtained using spontaneously immortalized capn4<sup>P/P</sup>; cre MEFs (l-MEFs) and their lentiviral-capn4 rescued counterparts (Fig. 4). After 3 μg/ml TN or 5 μM TG treatment for 24 h, apoptosis was measured by trypsin blue staining of total dead cells (Fig. 4A), or annexin-V and 7-AAD staining of apoptotic cells (Fig. 4B). Fewer dead cells were seen in primary capn4<sup>P/P</sup>; cre MEFs compared with the primary capn4<sup>+/+</sup>; cre MEFs in response to either TN or TG treatment. Fewer dead cells were also observed in the immortalized capn4<sup>P/P</sup>; cre MEFs compared with their capn4 rescued counterparts after TG incubation. Apoptosis was also independently assessed by flow cytometry, where cells with a sub-G1 content of PI-staining DNA were considered apoptotic (Fig. 5). This analysis also showed that calpain deficiency in either primary or immortalized MEFs correlated with resistance to ER stress-induced apoptosis. These observations support a permissive role for calpain in ER stress-induced apoptosis.

**Calpain-deficient Cells Display Defective ER Stress-induced Activation of the Caspase-12, -9, -3 Cascade.—** Caspase-12 plays a pivotal role in ER stress-induced apoptosis (15). Like other initiator caspases, activation of pro-caspase-12 requires the cleavage of a short inhibitory peptide. Active caspase-12 can then directly activate downstream caspase-9 independently of cytochrome c release from the mitochondria (9, 16). Active caspase-9 in turn activates the executioner caspase-3, leading to apoptosis. Caspase-12-null cells were resistant to ER stress-induced apoptosis, and in vitro experiments showed that m-calpain could mediate conversion of pro-caspase-12 into an active form by cleavage of the N-terminal pro-peptide regulatory sequence (9). We therefore asked if ER stress-induced caspase-12 cleavage and activation was compromised in calpain-deficient cells. In primary capn4<sup>P/P</sup>; cre MEFs, the 55-kDa caspase-12 was quickly cleaved in response to TN treatment, but such cleavage was delayed in the capn4<sup>P/P</sup>; cre MEFs (Fig. 6A). This was also seen after TG treatment of primary MEFs (Fig. 6B). In the immortalized capn4<sup>P/P</sup>; cre MEFs, caspase-12 cleavage was apparent as early as 6 h and essentially complete after 12 h of TG treatment (Fig. 6C, left lanes). In contrast, TG-induced caspase-12 cleavage was significantly attenuated in the immortalized capn4<sup>P/P</sup>; cre cells (Fig. 6C, middle lanes). Interestingly, the rescued immortalized capn4<sup>P/P</sup>; cre MEFs demonstrated considerably lower levels of caspase-12 expression, and this was further diminished upon TG treatment (Fig. 6C, right lanes). Collectively, these results strongly support a role for calpain in ER stress-induced caspase-12 activation.

Because active caspase-12 can directly cleave and activate caspase-9, which in turn can activate caspase-3, we next explored the requirement for calpain in ER stress-induced activation of caspase-9 and caspase-3, as well as cleavage of the caspase-3 substrate, PARP. In the primary capn4<sup>P/P</sup>; cre MEFs, caspase-3 was quickly activated after TN treatment, while it appeared much later and to a lesser extent in the capn4<sup>P/P</sup>; cre MEFs (Fig. 6A). As expected, PARP cleavage followed precisely the activation kinetics of caspase-3. During TG treatment, weaker activation of the caspase-9/caspase-3/PARP cascade was observed in primary capn4<sup>P/P</sup>; cre MEFs relative to capn4<sup>+/+</sup>; cre MEFs (Fig. 6B). In immortalized capn4<sup>P/P</sup>; cre MEFs, which retain normal calpain small subunit expression and calpain activity, pro-caspase-9 was cleaved to the active form in response to TG treatment (Fig. 6C, left lanes). In the immortalized capn4<sup>P/P</sup>; cre MEFs, only a small amount of cleaved caspase-9 was seen (Fig. 6C, middle lanes). In immortalized capn4<sup>P/P</sup>; cre MEFs that had been rescued with the capn4 lentivirus (I-rescue) we observed a partial rescue of cleaved caspase-9, and this was apparent as early as 12 h after TG exposure (Fig. 6C, right lanes). The kinetics of caspase-3 cleavage and activation correlated with those of caspase-9 activation in these
immortalized MEFs, with maximal cleavage of both caspase-3 and PARP at 24 h in calpain-expressing cells, compared with 30 h in the calpain-deficient cells (Fig. 6C).

**Calpain Does Not Regulate the Activation of Bcl-2 Family Members by Direct Cleavage during ER Stress**—Although caspase-12 is thought to be the initiator of ER stress-induced apoptosis, it was recently recognized that activation of ER residing Bax/Bak proteins occurs upstream of caspase-12 cleavage, because caspase-12 was not cleaved in Bax/Bak double knock-out cells (37, 38). Although the mechanism of Bax/Bak activation is unknown, a role for BH3-only proteins has been proposed (39). Our data strongly suggested that calpain has a direct role in cleaving caspase-12; however, it was still possible that calpain has additional upstream roles, perhaps at the level of Bax/Bak activation. Calpain has been implicated in the regulation of several different Bcl-2 family members through direct cleavage during apoptosis (1). For example, calpain-mediated cleavage of Bcl-xL in glial cells is proposed to convert an anti-apoptotic molecule into a pro-apoptotic one during ER stress-induced apoptosis (9). We therefore analyzed the protein levels of members of the Bcl-2 family in immortalized capn4/cre cells and their capn4 rescued counterparts during ER stress-induced apoptosis (Fig. 7). However, we did not observe any reproducible differences in either the steady state or post TG-treatment levels of Bax, Bad, Bak, Puma, Bok, Bik, Bim, Bcl-2, Bcl-XL, and Bcl-XS (normal and deaminated forms) (40). This suggested that μ- and m-calpain did not directly cleave these proteins in MEF cells during ER stress.

**Calpain Potentiates Sustained ER Stress-induced Activation of the ASK1 MAP Kinase Pathway**—ER stress can induce JNK activation through activation of IRE1. Interestingly, JNK activation was undetectable in primary capn4/cre MEFs whereas both the p46 and p54 JNK isoforms were activated in primary capn4+/cre MEFs (Fig. 8A). Consistent with this, phosphorylation of the downstream JNK target c-Jun, and the upstream JNK activator MKK4 were both compromised in calpain-deficient primary capn4/cre MEFs. Lastly, we examined the MKK4 kinase, ASK1 (41). Phosphorylation at the ASK1 activation loop threonine 845, which is diagnostic for the active form of ASK1, was induced by TG in primary capn4+/cre MEFs but not in capn4/cre MEFs (Fig. 8A). A similar defect in the activation of ASK1, MKK4, JNK, and c-Jun was observed after TG treatment in immortalized capn4P/P; cre MEFs (Fig. 8B). This defect in the AKT/MKK4/c-Jun ER stress cascade was also apparent in immortalized capn4P/P; cre MEFs, where it was effectively rescued using the capn4 rescue lentivirus (Fig. 8C). These observations clearly implicate calpain as an important participant in the upstream regulation of the ER stress-induced ASK1/c-Jun pathway. Other major signaling pathways including ERK, p38, NFκB, and Akt were not obviously affected by calpain deficiency in these immortalized MEFs (Fig. 8C).

**Calpain Deficiency Does Not Affect Transcriptional Responses to ER Stress**—We next examined some of the major transcriptional responses to TG-triggered ER stress, including genes encoding the chaperone protein Bip, and the transcription factors Xbp-1 and Chop. Immortalized capn4P/P; cre MEFs and their capn4 rescued counterparts displayed no differences in Bip, Xbp-1, or Chop transcript levels before or after TG treatment; nor was there any difference in the levels of the alternatively spliced Xbp-1 transcripts (Fig. 9A). This indicated that calpain did not affect IRE1 activation and its subsequent release from the ER because mRNA levels of the ATF6 target gene Bip, and the Perk target gene Chop were indistinguishable between immortalized capn4P/P; cre MEFs and their capn4 rescued counterparts (Fig. 9A). However, we did note that resting Bip protein levels (and possibly mRNA levels) were slightly higher in immortalized capn4P/P; cre MEFs after transduction with the capn4 rescue lentivirus (Fig. 9A and B). No difference was observed between the primary capn4+/cre; and capn4P/P; cre MEFs (Fig. 9C).
DISCUSSION

In this study, we provide novel genetic evidence that calpain participates in the apoptosis response to ER stress. This analysis validates the proposed involvement of calpain in activation of caspase-12, and it also provides novel evidence implicating calpain in activation of ASK1 and the JNK stress response pathway. Although calpain has been implicated in cleavage and regulation of Bcl-2 family members, our analysis does not support such a role in the context of ER stress in MEFs.

MEFs from the conditional capn4 knock-out mice represent a powerful in vitro system to explore the cellular functions of calpain. The capn4 knock-out MEFs lacked any detectable \( \mu \)- and m-calpain activities or small subunit expression, and this activity and expression was rescued upon lentiviral transduction with capn4 cDNAs (Fig. 1). This genetic model system is superior to interfering RNA methods, which do not achieve complete inhibition of calpain expression, and protease inhibitors which may lack specificity or the ability to completely inhibit calpain activity.

 Upon exposure to calcium in vitro, calpain subunits undergo autocleavage, which facilitates activation, and ultimately leads to calpain degradation (1). In vivo, ER stress causes calcium efflux from the ER, and this has been reported to activate calpain (42–44). It has also been suggested that this might involve calpain translocation to the ER (35). Calpain was reported to have a wide subcellular distribution including the ER and perinuclear regions (35, 36). In our study, TN or TG treatment quickly elevated intracellular free calcium levels in NIH3T3 fibroblasts, and this was followed by accumulation of m-calpain at the ER within 2 h (Fig. 2). This suggested that calpain translocation to the ER might be linked to regionally elevated calcium concentrations. We also found that prolonged treatment with TN or TG correlated with depletion of \( \mu \)-80 and m-80 protein levels (Fig. 3). Since activated calpain rapidly undergoes auto-proteolysis, the observed accumulation of calpain at the ER and subsequent depletion of large subunits during ER stress argues that calpain is massively activated at the ER in response to calcium release. A pro-apoptotic role for calpain at the ER was strongly suggested by the observation that ER stress-induced apoptosis was significantly attenuated in calpain-deficient MEFs (Figs. 4 and 5). The increased ER localization of calpain after TN or TG treatment might give it access to ER-residing proteins, including caspase-12. Calpain inhibitors were previously shown to attenuate ER stress-induced caspase-12 activation, and in vitro evidence for calpain-mediated cleavage and activation of caspase-12 provided an attractive model to explain this relationship (9). Our observations in calpain-deficient MEFs now provide compelling genetic data to support this hypothesis. Lack of calpain correlated with a defect in TG- or TN-induced activation of caspase-12, and \( \mu \) and m-calpain rescued MEFs before and after TG treatment for the indicated times (C). Immunoblotting analysis of p-ERK, p-p38, p-AKT, p-\( \mu \)-B, and p-ASK1, p-MKK4, p-JNK, and p-Jun in immortalized I-capn4P/P; cre and capn4 rescued MEFs before and after TG treatment for the indicated times (C).
targeted Bak (37, 38). Bax activation during ER stress might involve displacement of Bcl-2 by BH3-only proteins, such as Bad (47) or Bim (39), leading to the activation of caspase-12. Calpains have also been implicated in cleavage of Bcl-2 family members including Bax (8, 48, 49) and Bcl-2 (9). This raised the possibility that activation of caspase-12 might involve a role for calpain in cleavage of Bcl-2 family members. However, our analysis of a number of these proteins failed to reveal any difference in their steady state levels or ER stress-induced levels in calpain-deficient cells.

Our data support the hypothesis that calpain might serve as a link from Bax activation to caspase-12 activation. ER stress-activated Bax might promote calcium release from the ER by blocking the inhibitory role of Bcl-xL on InsP3R-mediated calcium release (50) thus activating ER stress-activated Bax during ER stress might involve a role for calpain in cleavage of Bcl-2 family members. However, our analysis of a number of these proteins failed to reveal any difference in their steady state levels or ER stress-induced levels in calpain-deficient cells.

Calpain Regulates ER Stress-induced Apoptosis

Calpain deficiency did not affect transcriptional responses to ER stress. A, TG-induced transcription of mRNAs encoding the chaperone protein Bip, and the transcription factors Xbp-1 and Chop were analyzed using semi-quantitative RT-PCR in immortalized IRE1P; cre MEFs and the corresponding capn4 rescued MEFS. B, TG-induced changes in Bip protein levels were assessed by Western blotting in immortalized IRE1P; cre MEFS and the corresponding capn4 rescued MEFS. C, TG-induced changes in Bip protein levels was also assessed by Western blotting in primary P-capan4P; cre and P-capn4P; cre MEFS.

In conclusion, using gene targeted capn4 knockout MEFS we have conclusively demonstrated an important pro-apoptotic role for the ubiquitous calpains in the ER stress pathway. Our analysis suggests that Bax-induced calcium release from the ER leads to the recruitment and activation of calpain, which is responsible for the activation of caspase-12, and subsequent activation of caspase-9 and caspase-3. We also provide novel evidence implicating calpain in the activation of ASK1, and subsequent activation of MKK4 and JNK. It remains to be determined what upstream calpain targets are responsible for ASK1 activation.

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