Ubiquitous Calpains Promote Both Apoptosis and Survival Signals in Response to Different Cell Death Stimuli*

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Yinfei Tan†, Chao Wu‡, Teresa De Veyra‡, and Peter A. Greer†§

From the ‡Division of Cancer Biology and Genetics, Queen’s University Cancer Research Institute and §Department of Pathology and Molecular Medicine, Queen’s University, Kingston, Ontario K7L-3N6, Canada

The μ- and m-calpain proteases have been implicated in both pro- or anti-apoptotic functions. Here we compared cell death responses and apoptotic or survival signaling pathways in primary mouse embryonic fibroblasts (MEFs) derived from wild type or Capn2- and Capn4-/- mice which lack both μ- and m-calpain activities. Capn2-/- MEFs displayed resistance to puromycin, camptothecin, etoposide, hydrogen peroxide, ultraviolet light, and serum starvation, which was consistent with pro-apoptotic roles for calpain. In contrast, Capn4-/- MEFs were more susceptible to staurosporine (STS) and tumor necrosis factor α-induced cell death, which provided evidence for anti-apoptotic signaling roles for calpain. Bax activation, release of cytochrome c, and activation of caspase-9 and caspase-3 all correlated with the observed cell death responses of wild type or Capn4-/- MEFs to the various challenges, suggesting that calpain might play distinct roles in transducing different death signals to the mitochondria. There was no evidence that calpain cleaved Bcl-2 family members proteins that regulate mitochondrial membrane permeability including Bcl-2, Bcl-xl, Bad, Bak, Bid, or Bim. However, activation of the phosphatidylinositol 3 (PI3)-kinase/Akt survival signaling pathway was compromised in capn4-/- MEFs under all challenges regardless of the cell death outcome, and blocking Akt activation using the PI3-kinase inhibitor LY294002 abolished the protective effect of calpain to STS challenge. We conclude that the anti-apoptotic function of calpain in tumor necrosis factor α- and STS-challenged cells relates to a novel role in activating the PI3-kinase/Akt survival pathway.

Apoptosis provides an important mechanism to eliminate cells during development and ontogeny and to maintain tissue homeostasis in the adult organism. Apoptosis can be induced by the activation of plasma membrane death receptors (extrinsic pathway) or by various stimuli that disturb intracellular functions required to maintain the integrity of the mitochondrion and endoplasmic reticulum (intrinsic pathway). Defective apoptotic cell death contributes to autoimmune diseases and tumorigenesis, whereas increased apoptosis is often associated with neurodegenerative diseases such as Alzheimer, Huntington, and Parkinson diseases (1).

Biochemical and genetic studies have established that apoptosis can be initiated and executed by caspases, a family of cysteine proteases (2). Initiator caspases, like caspase-8 and -10, are activated by death receptors including TNFα2 and FAS, whereas caspase-12 is activated by endoplasmic reticulum stress, and caspase-9 is activated at the apoptosome by a mechanism involving Apaf-1 and cytochrome c (3, 4). Initiator caspases can in turn activate executioner caspases, such as caspase-3 and caspase-7. Other important players include the pro-apoptotic proteins Bax and Bak, which control mitochondrial membrane permeability (MMP) and the release of cytochrome c, which is required to activate caspase-9 at the apoptosome. In the resting state Bax and Bak are under the suppression of Bcl-2. Upon death signaling, BH3-only proteins including Bim, Bad, and Bid are activated by transcriptional regulation or post-translational modification, which directly prompt Bax and Bak activation, leading to MMP and release of cytochrome c (4, 5). Calcium also plays a pivotal role in apoptosis. The release of calcium from the ER triggered mitochondria calcium overload, which directly contributed to MMP and release of cytochrome c, second mitochondria-derived activator of caspase, and apoptosis-inducing factor (6). Bax and Bak also control the resting calcium concentration in the ER. Cells deficient in Bax and Bak were found to have a reduced resting ER calcium concentration that resulted in decreased uptake of calcium by mitochondria after calcium release from the ER and lack of apoptosis (7). Interestingly, released cytochrome c was shown to bind to inositol 1,4,5-trisphosphate receptors on the ER resulting in sustained, oscillatory cytosolic calcium increases. This resulted in augmented cytochrome c release, which amplified the apoptotic signal in a feed-forward fashion (8). On the other hand, anti-apoptotic Bcl-xl interacts with the inositol 1,4,5-trisphosphate receptors, which reduces ER calcium content and stimulates mitochondrial energetics, therefore protecting cells against apoptosis (9). Besides prompting cytochrome c release, elevated cytosolic calcium levels exert other effects on apoptosis. For example, calcium activates calcinerin, which dephosphorylates p-Bad and causes its translocation from the cytosol to the mitochondria leading to activation of Bax (10).

The ubiquitous calpains can also be activated by calcium influx, and they too participate in many steps of apoptosis. Calpains are a family of calcium-dependent intracellular cysteine proteases, including the ubiquitously expressed μ- and m-calpains as well as a number of distinct tissue-specific calpains (for review, see Ref. 11). μ- and m-calpains are both 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 capn4. Biochemical analysis has revealed that the small subunit is indispensable for μ- and m-calpain, and genetic disruption of capn4 abolished the activity of both calpains (12). Interestingly, in vitro m-calpain activity requires calcium in the millimolar range, whereas micromolar concentrations...
activate μ-calpain. These calcium levels are not likely to be achieved in vivo; however, highly localized concentrations of calcium might occur transiently in close proximity to ion channels or the ER during stress. Calpains are also negatively regulated by the endogenously expressed peptide inhibitor calpastatin, and overexpression of recombinant calpastatin has been used to inhibit endogenous calpain activity (14).

Calpain has emerged as an important player in cell death signaling. Partial cleavage of pro- or anti-apoptotic proteins might activate or inactivate, respectively, putative substrates including p53 (15), Bcl-2 (16), Bcl-xl (16, 17), Bid (16, 18), Bax (19), caspase-3 (20–22), caspase-7, -8, and -9 (23), caspase-12 (17), and NFκB (24). For example, calpain cleavage of p53 protected cells from DNA damage-induced apoptosis, whereas calpain-cleaved p18 Bax has more potent pro-apoptotic activity (19). Controversially, calpain cleavage of caspase-9, -8, -7, and -3 attenuates their activity during apoptosis in many cell types (21–23), whereas other reports suggested that initial cleavage of caspase-3 by m-calpain facilitated its subsequent cleavage into active forms in neurons (20).

Many of the studies described above relied on small molecule inhibitors that lack complete calpain specificity on overexpression of calpain or calpastatin or on in vitro proteolysis of the putative calpain substrates. All of these approaches have inherent pitfalls that have confused our understanding of the role of calpain in cell death signaling. To address the physiologic functions of calpain during apoptosis, we have subjected primary embryonic fibroblasts from wild type and capn4−/− knock-out mice (12) to a wide range of cell death stimuli and examined several key apoptosis and survival pathways. Our results show that calpain plays pro-apoptotic roles in responses to puromycin, camptothecin, etoposide, hydrogen peroxide, ultraviolet light, and serum starvation and that it plays anti-apoptotic roles in cells challenged with TNFα or staurosporine (STS). Activation of the phosphatidylinositol 3-kinase/AKT survival pathway was correlated with calpain expression in all cell death challenges. This provides novel evidence for an important role for calpain in regulation of a key pro-survival pathway.

**MATERIALS AND METHODS**

**Chemicals and Antibodies**—STS, TNFα, etoposide, camptothecin, hydroxyurea (HU), cyclohexamide, puromycin, propidium iodide, and acridine orange were purchased from Sigma. CcαCeramide was from Alexis (Lausen, Switzerland). ALLN and MDL28170 were from Calbiochem. JC-1 and reactive oxygen species detection kit were from Molecular Probes, and annexin-V-fluorescein isothiocyanate (FITC) was from Calbiochem. Antibodies against caspase-8, -9, -3, and -1 were from Cell Signaling Technology. Antibodies against caspase-2, -6, and -7 were from Santa Cruz Biotechnology, and Bcl-2, Bax, Bcl-xL, and Bcl-XL were from Alexis. Antibodies against caspase-2, -6, and -7 were from Cell Signaling Technology. Antibodies against caspase-8, -9, -3, and -1 were from Cell Signaling Technology. Antibodies against caspase-2, -6, and -7 were from Cell Signaling Technology. Antibodies against caspase-8, -9, -3, and -1 were from Cell Signaling Technology. Antibodies against caspase-2, -6, and -7 were from Cell Signaling Technology.

**Cell Culture**—Primary mouse embryonic fibroblast (MEF) cultures were established from E10.5 wild type and capn4−/− embryos. The yolk sacs, heads, and internal organs were isolated and used for genotyping by Southern blot hybridization as described previously (12). Carcasses were treated with 0.25% collagenase in 20% fetal bovine serum in phosphate-buffered saline for 30 min at 37 °C, and after centrifugation, the cells were suspended in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal bovine serum, antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml), and 2 mM L-glutamine. Primary MEFs were used before passage 4.

**FIGURE 1.** Capn4−/− MEFs lack μ- and m-calpain activities. A, zymogram analysis of μ- and m-calpain activities in capn4−/− MEFs (wt, wild type). B, Western blotting analysis of the 80-kDa m-calpain large subunit (m-80) and the 28-kDa small subunit (CAPN4). C, Western blotting analysis of the 80-kDa μ-calpain large subunit (μ-80).

**Apoptosis Analysis**—MEFs were seeded at 3 × 10^5/6-cm plate and cultured overnight before use. On the following day cells were treated with 10 ng/ml TNFα plus 200 ng/ml cyclohexamide, 4 μM puromycin, 5 μM camptothecin, 100 μM etoposide, 2 mM HU, 80 J/m2 UV, 500 mM STS, and 5 μM ceramide, serum starvation, or H2O2 (1 μM for the first 1 h and maintained at 100 nM thereafter) for the indicated times. Apoptosis was measured by 0.2% trypan blue staining or flow cytometry analysis of phosphatidylinositol 3-phosphate-phosphatidylcholine labeled annexin-V and 7-AMCA staining according to the Clontech product manual (26). Apoptotic DNA condensation was measured as the sub-G1 DNA peak stained by propidium iodide as described previously (27).

**Analysis of Lysosome Stability**—Cells were exposed to 5 μg/ml acridine orange for 15 min under standard culture condition, then harvested by trypsin treatment and washed twice with medium as described (28, 29). Red fluorescence was measured immediately by flow cytometry. Cells with decreased red fluorescence (pale cells) were quantified.

**Western Blotting**—Cells were collected at the indicated times post-treatment. Medium was aspirated, and cells were washed twice with ice-cold phosphate-buffered saline. Cell monolayers were incubated 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) on ice for 15 min. Lysates were recovered by scraping and centrifuged to remove particulate debris. Protein content was determined by the Bradford method. Samples (20 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Immunoblots were incubated with primary antibodies at 4 °C overnight, then incubated with secondary antibody conjugated with horseradish peroxidase for 60 min at room temperature. Signals were then developed by enhanced chemiluminescence using the ECL kit (Amersham Biosciences).

**Release of Cytochrome c and Activation of Bax**—Monolayers of 2 × 10^6 cells were washed twice in ice-cold phosphate-buffered saline, then harvested by scraping and centrifugation at 800 × g for 5 min. The cell pellet was suspended in 600 μl of 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 (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin). After 30 min of incubation on ice, cells were subjected to 80 strokes with a Dounce homogenizer and spun for 60 min at 14,000 × g. Supernatants were analyzed for cytochrome c release by Western blotting (30). Immunoprecipitation of active Bax was as described (31). Briefly, cells were lysed using CHAPS lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% CHAPS, plus protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin)), then centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was immunoprecipitated using monoclonal anti-Bax 6A7 and protein G-agarose and washed over-
night at 4 °C. The complexes were then washed five times with CHAP lysis buffer and subjected to SDS-PAGE and immunoblotting with a polyclonal anti-Bax antibody, as described above.

In Vitro Analysis of \( \text{H9262} \) - and \( m \)-Calpain Activities—Calpain activities in cell extracts were detected by casein zymography in a standard Tris-glycine system (25). 40 \( \mu \)g of protein was resolved on 8% nondenaturing polyacrylamide gels containing 1.5 mg/ml casein. Calpain was activated by incubating the gels overnight with 5 mM Ca\(^{2+} \), and its activity was visualized as areas cleared of casein after staining with Coomassie Brilliant Blue.

Statistical Analysis—Results were expressed as the means ± S.D. of at least three independent experiments performed in triplicate. Statistical analysis was performed using Student’s \( t \) test, with a level of significance set at \( p < 0.05 \).

RESULTS

Ubiquitous Calpains Have Both Pro- and Anti-apoptotic Functions—Calcium-regulated \( \mu \)- and \( m \)-calpain activities have been implicated in many cellular signaling pathways, including apoptosis. However, much of the literature supporting this hypothesis relies on pharmacological inhibitors of questionable specificity or effectiveness. To avoid those problems we employed primary MEFs derived from a mouse knock-out model of calpain (12). Targeted disruption of the \( \text{capn4} \) gene abolished expression of the small regulatory subunit (Fig. 1, panel B). Casein zymography analysis of calpain activity demonstrated loss of both \( \mu \)-calpain and \( m \)-calpain activities in \( \text{capn4}^{+/−} \) MEFs (Fig. 1A). Immunoblotting analysis showed that steady state levels of both \( \mu \)-80 and \( m \)-80 catalytic subunits were dramatically reduced in \( \text{capn4}^{+/−} \) MEFs (Fig. 1, panels C and B, respectively). These observations support the
hypothesis that the capn4-encoded 28-kDa subunit is required for the stability and activity of the μ-calpain and m-calpain catalytic subunits, μ-80 and m-80 respectively, and this demonstrates that capn4−/− MEFs are devoid of detectable calpain activity.

These primary wild type and capn4−/− MEFs were then used to explore the involvement of the ubiquitous calpains in response to challenges with different death stimuli (Fig. 2). Statistically significant differences in cell death responses, as assessed by trypan blue staining, were seen with a number of different challenges (Fig. 2A). The translation inhibitor puromycin, the topoisomerase I inhibitor camptothecin, the topoisomerase II inhibitor etoposide, serum deprivation, ultraviolet light (UV), or hydrogen peroxide (H2O2) induced significantly more cell death in wild type than in capn4−/− MEFs. In contrast, more cell death occurred in capn4−/− than wild type MEFs after challenge with either the death receptor ligand TNFα or the broad-spectrum kinase inhibitor STS. These results indicated that calpain plays pro-apoptotic roles in apoptosis induced by growth factor deprivation, DNA damage, reactive oxygen species, and translation inhibition but that calpain plays anti-apoptotic roles in apoptotic signaling induced by TNFα or STS.

Analysis of PARP cleavage was also used to assess activation of the executor caspase-3 in response to these challenges (Fig. 2, panels B–G). The degree of PARP cleavage in response to all challenges correlated precisely with the observed cell death response. Although HU-induced cell death did not show a statistically significant difference, there was an apparent increase in PARP cleavage in wild type relative to capn4−/− MEFs. No significant cell death was observed in wild type or capn4−/− MEFs in response to ceramide treatment, and this correlated with the observed absence of PARP cleavage.

Phosphatidylserine exposure on the outer leaflet of the plasma membrane and DNA degradation were also examined as indicators of cell death responses (32). In response to puromycin, wild type MEFs displayed reduced annexin-V association with external phosphatidylserine and fewer numbers of cells with a sub-G1 DNA content in comparison with capn4−/− MEFs (Fig. 3, panels A and B, respectively). The opposite responses were observed in response to TNFα or STS treatment.

**Calpain Functions Upstream of Apoptotic Mitochondrial Cytochrome c Release**—We next examined release of cytochrome c from the mitochondria in response to challenge with puromycin, TNFα, or STS (Fig. 4, panel A). The puromycin-induced release of cytochrome c was attenuated in capn4−/− MEFs relative to wild type cells, and this correlated with reduced activation of Bax, caspase-9, caspase-3, and reduced PARP cleavage. In contrast, cytochrome c release in response to TNFα or STS treatment was enhanced in the capn4−/− MEFs relative to wild type cells. Consistent with this, we observed more activation of Bax, caspase-9, and caspase-3 and more PARP cleavage in TNFα- or STS-challenged capn4−/− MEFs compared with wild type cells.
These results indicated that calpain involvement in the regulation of signals leading to MMP and caspase-3 activation are pro-apoptotic in the case of puromycin but anti-apoptotic in the case of TNFα or STS challenges. Furthermore, these results suggest roles for calpain at points upstream of MMP. Calpains have been implicated in the cleavage of a number of Bcl-2 family members that regulate MMP; however, we observed no evidence for this in response to puromycin, TNFα, or STS. The relative levels of Bcl-2, Bcl-xL, Bad, Bak, Bid (Fig. 4, panel B), and Bim (data not shown) were indistinguishable between wild type and capn4−/− MEFs during these cell death challenges. This suggested that instead of directly cleaving Bcl-2 family members, including the BH3-only proteins that regulate Bax activation, calpain might participate further upstream in signaling pathways that control the modification and activation of BH3 only proteins. Alternatively, calpain might participate in other pathways that integrate cell death and survival signals.

For example, Bad and Bim lose their pro-apoptotic effects through phosphorylation mediated by either ERK (33, 34) or Akt (35), whereas Noxa and Puma are controlled primarily at the transcriptional level (6).

**Calpain Modulates Survival and Apoptotic Signaling Pathways during Apoptosis—**ERK (p42/44), AKT, and NFκB pathways typically contribute to cell survival, whereas stress signaling pathways such as INK and p38 can promote apoptosis (36–40). We, therefore, assessed the activation status of these pathways in wild type and capn4−/− MEFs in response to the various cell death stimuli (Fig. 5). Puromycin treatment activated INK and AKT in wild type but not in capn4−/− MEFs, whereas ERK activation was similar in both cell types, and no significant phosphorylation of IkBα was noticed in either cell type (Fig. 5, panel A). A similar trend of reduced activation of INK and AKT in capn4−/− MEFs was observed in response to camptothecin, etoposide, UV, ceramide, and H2O2, suggesting that calpain participates in activation of both INK and AKT in response to these agents. ERK activation was also compromised in capn4−/− MEFs in response to camptothecin, UV, ceramide, H2O2, starvation, and H2O2, which also implicated calpain in the activation of ERK in response to these agents. A notable exception to these trends was seen during H2O2 treatment, where capn4−/− MEFS displayed a more substantial activation of INK, in particular the p54 isoform. Interestingly, H2O2 also provoked a significant, yet transient phosphorylation of IkBα in capn4−/− MEFS but had little effect in wild type cells (Fig. 5, panel D).

These signaling pathways were also examined in response to TNFα and STS challenge (Fig. 5, panels E and F, respectively). TNFα triggered ERK activation in capn4−/− MEFS, but not in wild type cells, whereas STS elicited similar ERK activation in both cell types. TNFα and STS induced very strong activation of AKT relative to puromycin and other death stimuli, and this activation was substantially reduced in capn4−/− MEFS. We also observed a greater magnitude and more prolonged TNFα- and STS-induced AKT activation, which was significantly increased in wild type relative to capn4−/− MEFS. The degree of TNFα- and STS-induced INK and p38 activation was similar in wild type and capn4−/− MEFS. Interestingly, no p38 activation was detected when cells were challenged with cell death stimuli other than TNFα or STS (data not shown). Collectively, these data suggest that calpain plays an important role in activation of the AKT survival pathway and that this might be a much more relevant survival pathway in cells challenged with TNFα and STS than in cells challenged with puromycin or other death stimuli.

**Phosphatidylinositol 3′-Kinase Inhibition Abolished Calpain Anti-apoptotic Function during STS-induced Apoptosis—**AKT exerts anti-apoptotic functions by phosphorylating and inactivating pro-apoptotic factors including Bad and forkhead transcription factors AFX, FKHR, and FKHRL1 (41, 42). Persistent AKT activation was observed in TNFα- and STS-treated wild type cells (Fig. 5, panels E and F), and there was also a positive correlation between calpain expression and AKT activation in response to several other death stimuli (Fig. 5). Therefore, we next asked if blocking AKT activation by inhibiting the upstream regulator phosphatidylinositol 3′-kinase could abolish the protective effect of calpain to challenge with TNFα or STS. The phosphatidylinositol 3′-kinase inhibitor LY294002 significantly increased TNFα-induced apoptosis in both capn4−/− (p = 0.021) and wild type MEFS (p = 0.011), as shown by trypan blue staining and PARP cleavage (Fig. 6, panels A and B, respectively). In contrast, LY294002 completely reversed calpain’s protective role in wild type cells during STS-induced apoptosis (p = 0.005) but had no effect on the degree of cell death in capn4−/− MEFS. This suggested that the protective effect in the case of STS might be due to calpain-mediated AKT activation. The effects of LY294002 on cell death responses also correlated with Bax activation and cytochrome c release (Fig. 6, panel C).

**Pharmacological Calpain Inhibitors Block Calpain-Dependent Pro- and Anti-apoptosis Signals—**We next tested the ability of two calpain inhibitors to interfere with the apparent anti- or pro-apoptotic roles of calpain. Unfortunately, none of the available inhibitors is completely specific for calpain. Calpain inhibitor I (ALLN) inhibits μ- and m-calpains, cathepsins B and L, the proteasome, and the NFκB pathway (43, 44), whereas calpain inhibitor III (MDL28170) can inhibit calpain and

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**FIGURE 4.** Activation of the mitochondrial apoptosis pathway. A, PARP cleavage, caspase-3 activation, caspase-9 activation, release of cytochrome c, and activation of Bax were assessed by Western blotting analysis in primary wild type (P-wt) and calpain deficient P-capn4−/− MEFS after treatment with puromycin (Puro), STS, and TNFα for the indicated times. con, control. B, Western blotting analysis of Bcl-2, Bcl-xL, Bak, Bad, and Bid, as indicated.
Significant differences in cell death responses were seen between wild type and capn4/H11002/H11002 MEFs in all treatments except STS plus ALLN and ALLN alone for 3 h (Fig. 7, panel A). In TNFα-induced apoptosis, where calpain appeared to have a predominantly anti-apoptotic role, both MDL28170 and ALLN augmented the cell death response in wild type MEFs (p = 0.039 and p = 0.032, respectively), but neither drug significantly affected the response in capn4/H11002/H11002 MEFs (Fig. 7, panel A). In STS-induced apoptosis, where calpain also appeared to promote survival, MDL28170 and ALLN again induced large increases in wild type MEF cell death (p = 0.0005 and p = 0.0037, respectively). In STS-challenged capn4−/− MEFs, MDL28170 also significantly enhanced cell death (p = 0.0006), but ALLN had no appreciable effect. During puromycin-induced cell death, where calpain appeared to be playing a pro-apoptotic role, ALLN actually increased apoptosis in wild type cells (p = 0.016), whereas MDL28170 enhanced puromycin-induced apoptosis in capn4−/− MEFs (p = 0.0037). Calpain inhibitors alone also promoted cell death, and although the magnitude of this effect was greater in wild type cells, it was also apparent in the capn4−/− MEFs, especially in the case of ALLN (Fig. 7, panel A). In all cases, PARP cleavage corresponded precisely with cell death, as assessed by trypan blue staining (Fig. 7, panels B–F).

This provided additional evidence for a role for calpain in promoting AKT activation. However, calpain inhibitors did not affect AKT activation in wild type cells after TNFα challenge (Fig. 7, panel B), and the differential AKT response to TNFα between wild type and capn4−/− MEFs was not as great as that seen with STS challenge (Fig. 5, compare...
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AKT response in panels E and F). This argues that calpain’s protective effect might be more strongly linked to the AKT response in cells challenged with STS than in cells treated with TNFα.

Because the calpain inhibitors used here promoted greater increases in cell death in response to TNFα and STS in wild type relative to capn4−/− MEFs, it argued that at least some of their effects are due to calpain inhibition. However, these drugs also had effects on capn4−/− MEFs, notably in the case of puromycin- or ALLN-induced cell death, so it is apparent that they must also have other targets besides calpain, and some of these targets play roles in cell death signaling in response to the TNFα, STS, and puromycin.

Calpain Deficiency Resulted in Slightly More Loss of Lysosome Instability but No Difference in p53 Activation in Response to STS Treatment—

FIGURE 6. Phosphatidylinositol 3-kinase inhibition reverses calpain’s protective role in STS-induced apoptosis. A, primary wild type (P-wt) and calpain deficient (P-capn4−/−) MEFs were treated with TNFα or STS in the presence or absence of 10 μM phosphatidylinositol 3-kinase inhibitor LY294002 (LY). A, the percentages of dead cells were determined by trypan blue staining. Shown is apoptotic cleavage of PARP in response to TNFα (B) or STS (C). con, control. Both full-length and cleaved PARP are indicated. C, PARP cleavage, cytochrome c release from mitochondria, and Bax activation in response to STS. Data points with statistically significant differences (p < 0.05) are indicated (*).

cells (Fig. 8, panel A). Puromycin and TNFα did not trigger lysosome leakage (data not shown). LY294002 reduced the degree of STS-induced lysosome leakage, suggesting an involvement of phosphatidylinositol 3'-kinase, but there was no significant difference in this response between wild type and capn4−/− cells. Thus, differences in lysosome instability did not account for calpain’s protective role in STS-induced apoptosis.

STS can inhibit ERK, C-terminal Src kinase (CSK), protein kinase A, casein kinase 1, casein kinase 2, c-Fgr, Lyn, and TPK-IIB/p38Syk (49); however, its ability to induce apoptosis through the intrinsic apoptotic pathway is poorly understood. We found that STS could also trigger p53 activation. Although p53 is a suspected calpain substrate under some conditions (15), we did not observe any differences in STS—(Fig. 8, panel A) or camptothecin-induced (data not shown) p53 levels between wild type or capn4−/− MEFs. This argued that differential regulation of p53 did not contribute to the greater sensitivity of capn4−/− MEFs to STS-induced apoptosis.

DISCUSSION

Calpains have been proposed to play pro-apoptotic roles in the context of several different cell systems and in response to a wide range of different death stimuli. Dexamethasone-induced death of thymocytes was delayed by the calpain inhibitors ALLN, MDL28170, E64d, and ZLLY-CHN2 (50). Calpain was suggested to play synergistic roles with caspases and the proteasome during neutrophil apoptosis (51). Calpastatin overexpression in human UV(r)-1 fibroblasts protected them against okadaic acid induced apoptosis and also reduced c-Jun hyperphosphorylation (52). Calpain inhibitor studies implicated the ER stress-response protein GRP94 as a calpain substrate during etoposide-induced cell death in Chinese hamster fibroblast K12, and knockdown of GRP94 expression in Jurket cells correlated with cell death (53). The calpain inhibitor calpeptin blocked Bax cleavage and activation in HL-60 cells during camptothecin-induced cell death, but calpeptin did not delay cell death in that study (54). Cycloheximide-induced cell death of human neutrophils was inhibited by the calpain inhibitors ALLN, PD150606, and PD151746, and depletion of calpastatin promoted neutrophil cell death; however, in this study FAS ligand-induced cell death was not affected by calpain inhibitors (55). Cell death induced by reovirus infection was inhibited with calpain inhibitors ALLN and PD150606 (56). Calpain was shown to be capable of cleaving and activating caspase-12 and converting Bcl-xl from an anti- to a pro-apoptotic effector during ER stress-induced apoptosis (17). Calpastatin overexpression in B cells inhibited receptor-induced apoptosis (57). Overexpression of μ-calpain in CHO cells correlated with greater sensitivity to thapsigargin or the calcium ionophore A23187, whereas calpastatin overexpression protected CHO cells against these ER stress-inducing treatments (14). Inhibitor studies in neutrophils suggested a calpain-dependent pro-apoptotic function during challenge with TNFα (58). Finally, we have recently shown that calpain has a pro-apoptotic function during ER stress that correlated with activation of caspase-12 and the apoptosis signal-regulating kinase 1/mitogen-activated protein kinase kinase 6/NK-c-Jun pathway.3

In contrast, there are only a few reports suggesting anti-apoptotic functions for calpain. TNFα induced a NFκB survival pathway in the murine fibroblast cell line WEHI164 that could be blocked with calpain inhibitor I (13). Experiments either overexpressing μ-calpain or calpastatin in CHO cells indicated that calpain could protect against TNFα-induced apoptosis (14). Ceramide-induced NFκB pro-survival signaling was shown to be calpain-dependent using calpain-deficient

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![Diagram](image-url)

**FIGURE 7.** Pharmacological calpain inhibitors did not precisely mimic genetic calpain deficiency and aggravated apoptosis in both a calpain-dependent and -independent fashion. Primary wild type (P-wt) and calpain deficient (P-capn4⁻/⁻) MEFs were treated with TNFα, STS, or puromycin in the presence or absence of ALLN (100 μM) or MDL28170 (50 μM) as indicated. A, the percentages of dead cells were assessed by trypan blue staining. Significant differences in cell death responses were seen between wild type and capn4⁻/⁻ MEFs in all treatments except STS plus ALLN and ALLN alone for 3 h. Other comparisons that yielded significant differences are indicated with lines and asterisks: B, PARP cleavage and AKT activation in response to TNFα and calpain inhibitor treatment. p-, phospho. C, PARP cleavage and AKT activation in response to STS and calpain inhibitor treatment. D, PARP cleavage and AKT activation in response to puromycin and calpain inhibitor treatment. con, control. E, PARP cleavage in response to treatment of MDL28170 for 24 h or preincubation of ALLN for 3 h and further incubation for 24 h. F, PARP cleavage in response to treatment of ALLN for the indicated times.

fibroblasts (24). Biochemical studies have suggested that calpain might be able to cleave and inactivate caspase-7, -8, and -9 (23).

The preceding review of the literature on the role of calpain in apoptosis underscores the complexity of this question. Most of these previous studies relied heavily on the use of pharmacological calpain inhibitors which lack specificity for calpain. There is also the issue of incomplete inhibition of calpain using these approaches, and this might also complicate the interpretation of studies using overexpression of calpastatin. Finally, biochemical studies showing the ability of calpain to cleave suspected substrates in vitro are clearly informative, but they do not establish that these events are occurring in vivo. Calpain is likely to be acting at the level of several different signaling pathways, and collectively, these contribute in a very significant way to the eventual outcome associated with different death stimuli.

In this study we have used primary fibroblasts from wild type or capn4⁻/⁻ mouse embryos, which lack both μ- and m-calpain activities. Significant differences in the response to a number of cell death stimuli were observed, and these differences implicated calpain in pro-apoptotic signals associated with puromycin, camptothecin, etoposide, hydrogen peroxide, ultraviolet light, and serum starvation. In contrast, calpain appears to play anti-apoptotic functions in the cellular responses to TNFα and STS. Activation of Bax, release of cytochrome c, and activation of caspase-9 and caspase-3 correlated precisely with the different apoptotic outcomes, but we could see no evidence for a role for calpain in cleavage of Bcl-2 family members that might explain this relationship. We, therefore, turned our attention to the upstream survival and stress pathways associated with the kinases ERK, JNK, AKT, and p38. AKT was the only kinase that showed a consistently positive correlation between calpain expression and challenge with all death stimuli tested. ERK activation did not display a consistent association with calpain expression but with the exception of the TNFα challenge, there was a trend toward more ERK activation in wild type cells relative to capn4⁻/⁻ MEFs. Interestingly, JNK activation correlated positively with calpain expression in cells challenged with puromycin, etoposide, camptothecin, and UV, where calpain has a pro-apoptotic role, but not with TNFα or STS, where calpain has an anti-apoptotic role. Collectively, these data suggest that the differential degree of activation of survival and death pathways, in particular AKT and JNK, respectively, might account for the differential responses to the various death stimuli between wild type and capn4⁻/⁻ MEFs. Thus, calpain might contribute to the activation of AKT and JNK to different degrees in response to different death stimuli. Puromycin-elicited signals might lead to a greater degree of calpain-dependent activation of JNK relative to AKT. In contrast, TNFα- and STS-induced signals might activate calpain in a fashion that is more likely to participate in activation of AKT than of JNK. Clearly these are speculative interpretations, and it remains to be determined what specific calpain targets are involved in AKT and JNK activation or how differential death stimuli channel calpain’s activity in different directions. One obvious possibility is that these different signals provoke different changes in intracellular calcium availability, leading to different degrees of calpain activation at different subcellular localizations. We have recently observed ER stress caused by thapsigargin or tunicamycin caused activation of calpain and its localization at the ER, and this led to activation of both caspase-12 and JNK. Furthermore, genetic calpain deficiency correlated with reduced ER stress-induced cell death.

In further support of this proposed involvement of calpain in AKT activation, we showed that pharmacological inhibition of the upstream activator phosphatidylinositol 3'-kinase did block the calpain-dependent survival
advantage to STS, which elicited the greatest activation of AKT. However, the calpain inhibitors used here (MDL28170 and ALLN) also had effects on capn4−/− MEFs, which indicated that not all their effects were due to calpain inhibition. Indeed, ALLN induced cell death in calpain-deficient cells and enhanced puromycin-induced cell death in calpain-deficient cells. This indicates that these compounds must also have other targets besides calpain, and some of these targets play roles in cell death signaling in response to the TNFα, STS, and puromycin.

Thus far we have not been able to identify calpain targets that might explain its role in regulation of the AKT pathway. However, based upon the observations reported here, we suggest that efforts to understand the role of calpain in cell death signaling should be expanded to include...
this important survival pathway. The degree of engagement of this pathway will clearly affect the outcome associated with cell death stimuli, including a wide variety of drugs currently used in cancer treatment. This suggests that inhibition of calpain might sensitize cancer cells to the killing effects of some therapeutic drugs.

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REFERENCES

1. Hengartner, M. O. (2000) *Nature* **407**, 770–776
2. Fuentes-Prior, P., and Salvesen, G. S. (2004) *Biochem. J.* **384**, 201–232
3. Kidd, V. J., Lahit, J. M., and Teitz, T. (2000) *Cell. Dev. Biol.* **11**, 191–201
4. Willis, S. N., and Adams, J. M. (2005) *Cell. Biol. Cell Res.* **304**, 437–444
5. Cory, S., and Adams, J. M. (2002) *Nat. Rev. Cancer* **2**, 647–656
6. Scorrano, L., Oakes, S. A., Opperman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korosmeyer, S. I. (2003) *Science* **300**, 135–139
7. Boehning, D., Patterson, R. L., Sedaghat, L., Gelbova, N. O., Kurosksi, T., and Snyder, S. H. (2003) *Nat. Cell. Biol.* **5**, 1051–1061
8. White, C., Li, C., Yang, J., Petrenko, N. B., Madesh, M., Thompson, C. B., and Foskett, J. K. (2005) *Nat. Cell. Biol.* **7**, 1021–1028
9. Jayaraman, T., and Marks, A. R. (2000) *J. Biol. Chem.* **275**, 6417–6420
10. Jayaraman, T., Mark, M. A., and Marks, A. R. (2000) *J. Biol. Chem.* **275**, 6417–6420
11. Chua, B. T., Guo, K., and Li, P. (2000) *Cell Biol. Cell Res.* **304**, 201–232
12. Chen, M., He, H., Zhan, S., Krajewski, S., Reed, J. C., and Gottlieb, R. A. (2001) *FASEB J.* **15**, 171–186
13. Chua, B. T., Guo, K., and Li, P. (2000) *J. Biol. Chem.* **275**, 5131–5135
14. Chua, B. T., Guo, K., and Li, P. (2000) *FASEB J.* **15**, 171–186
15. Claudio, E., Segade, F., Wrobel, K., Ramos, S., Bravo, R., and Lazo, P. S. (1996) *FASEB J.* **10**, 247–254