

Calpains Mediate p53 Activation and Neuronal Death Evoked by DNA Damage*

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DNA damage is an initiator of neuronal death implicated in neuropathological conditions such as stroke. Previous evidence has shown that apoptotic death of embryonic cortical neurons treated with the DNA damaging agent camptothecin is dependent upon the tumor suppressor p53, an upstream death mediator, and more distal death effectors such as caspases. We show here that the calcium-regulated cysteine proteases, calpains, are activated during DNA damage induced by camptothecin treatment. Moreover, calpain deficiency, calpastatin expression, or pharmacological calpain inhibitors prevent the death of embryonic cortical neurons, indicating the important role of calpain in DNA damage-induced death. Calpain inhibition also significantly reduced and delayed the induction of p53. Consistent with the actions of calpains upstream of p53 and the proximal nature of p53 death signaling, calpain inhibition inhibited cytochrome *c* release and DEVD-AFC cleavage activity. Taken together, our results indicate that calpains are a key mediator of p53 induction and consequent caspase-dependent neuronal death due to DNA damage.

DNA damage is a critical initiator of delayed neuronal death and is thought to play an important role in neurodegenerative processes and stroke (1–3). As an example, DNA damage is thought to occur prior to DNA fragmentation associated with the late apoptotic program following ischemic injury (4). In addition, DNA damage has also been implicated in excitotoxic damage and oxidative stress, two mechanisms widely associated with the pathogenesis of a variety of neurodegenerative conditions, including Parkinson's disease (5). However, the signaling events activated upon DNA insults are not fully understood.

Recent work has shown that the topoisomerase 1 inhibitor camptothecin causes apoptotic death of cultured cortical neurons (6–10). The tumor suppressor p53 is an important component of the numerous death pathways activated by DNA damage. We (11, 12) and others (13, 14) have shown that p53 is up-regulated prior to death commitment and is required for activation of the conserved death program consisting of Bax

translocation (12), cytochrome *c* release (10, 15), and caspase activation (10, 15). However, the mechanism by which p53 is activated by DNA damage in neurons is not fully understood.

Calpains are calcium-dependent neutral proteases that have been implicated in a variety of physiological and pathological conditions, including regulation of cell cycle progression, neuronal plasticity, and initiation of neuronal cell death (16). Calpains μ and m are the two most ubiquitously expressed forms of calpains, and they require interaction with a small regulatory calpain subunit encoded by the gene *capn4* to function properly. The importance of calpains is underscored by the observation that mice deficient in the calpain small regulatory subunit die embryonically, perhaps due to cardiovascular defects (17, 18). With respect to calpain involvement in neuronal death, pharmacological calpain inhibitors have been shown to protect neurons from a variety of death stimuli, including ischemic/excitotoxic insults, both *in vitro* (19–21) and *in vivo* (22–24). However, the inhibitors utilized are known to inhibit targets other than calpains, and, therefore, the role of calpain in neuronal death has been unclear and controversial.

The regulation of calpain is complex and includes a requirement for calcium (16), the endogenous cellular calpain inhibitor calpastatin (25), and translocation to membranous compartments (26, 27). Calpains are reported to modulate a wide variety of intracellular signaling pathways by targeted cleavage of substrate proteins, including the Cdk5 activator p35 (28, 29), the NF κ B inhibitor I κ B (30), immediate early genes such as c-Fos and c-Jun (31), and the structural proteins fodrin (32) and spectrin (33). Calpains are also thought to cleave and inactivate caspases, the executors of the death signal in apoptosis (34). The diversity of the substrates cleaved by calpain highlights the varied nature of calpain-mediated signals and demonstrates its ability to regulate various cell functions, possibly by modulating different proteins under different contexts.

We currently examined the requirement for calpain activation in the death of cultured cortical neurons evoked by DNA damage. Multiple lines of evidence based upon studies of neuronally differentiated calpain-deficient stem cells and calpastatin overexpression, as well as multiple pharmacological calpain inhibitors, strongly suggest the involvement of calpains in neuronal death induced by DNA damage. Moreover, we find that calpain inhibition reduces p53 activation and consequent mitochondrial death effector signals. This evidence implicates p53 as a critical step by which calpains transduce the death signal.

EXPERIMENTAL PROCEDURES

Materials—Camptothecin was obtained from Sigma. DEVD-AFC was purchased from Enzyme Systems Products (Dublin, CA). PD 150606 and MDL 28170 were obtained from Calbiochem.

Generation of Recombinant Adenovirus—The recombinant adenoviral vectors were constructed using the AdEasy system as described

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previously (35). Calpastatin (RNCast104), subcloned previously into a pAdLox (36), was excised using *Bam*HI and *Eco*RI. The sequence encoding an enhanced green fluorescent protein (EGFP)¹ was excised from pEGFP-C (Clontech) using *Afe*I and *Sal*I. Both fragments were ligated into pShuttle-CMV to produce pShuttle-CMV-EGFP-Calpastatin. Calpastatin and the control EGFP adenovirus were amplified in 293 cells and purified over CsCl gradients. For primary cell infections, virus (multiplicity of infection 10) was added to neural cultures at the time of plating.

Culture and Survival of Cortical Neurons—Mouse cortical neurons were cultured from embryonic day 15 mice as described previously (11). Neurons were plated into 24-well dishes (~200,000 cells/well) or 6-well dishes (2–4 million cells/well) coated with poly-D-lysine (100 μ g/ml) in serum-free medium (N2/Dulbecco's modified Eagle's medium (1:1) supplemented with 6 mg/ml D-glucose, 100 μ g/ml transferrin, 25 μ g/ml insulin, 20 nM progesterone, 60 μ M putrescine, and 30 nM selenium. One to two days after initial plating, the medium was supplemented with camptothecin (10 μ M) alone or with calpain inhibitors as indicated in the text and figures. At appropriate times of culture, cells were lysed, and the numbers of viable cells were evaluated. Briefly, cells were lysed in 200 μ l of cell lysis buffer (0.1 \times PBS, pH 7.4, containing 0.5% Triton X-100, 2 mM MgCl₂, and cetyltrimethylammonium bromide (0.5 g/100 ml), which disrupts cells but leaves the nuclei intact. Ten microliters of sample from each culture were loaded onto a hemacytometer, and the number of healthy intact nuclei was evaluated by phase microscopy. Nuclei that displayed characteristics of blebbing, disruption of nuclear membrane, phase-bright apoptotic bodies, and chromatin margination were excluded. All experimental points are expressed as a percentage of cells plated on day 0. Alternatively, cells were collected and analyzed for biochemical analyses as described below.

Culture and Survival of Stem Cell-derived Neurons—Mice heterozygous deficient for the small subunit of calpain (Capn4^{+/-}) were bred, and the embryos were isolated at embryonic day 10.5. Mouse forebrain stem cells were cultured from embryonic telencephalons as described previously (Ref. 37; modified). Embryos were genotyped as described previously (17). Stem cells were plated at a density of 5 \times 10⁴ cells/ml and allowed to form neurospheres in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 20 μ g/ml basic fibroblast growth factor as described previously (37). To passage, cells were triturated to obtain a single cell suspension using a flame-polished glass pipette. To differentiate the neural stem cells, they were plated into 24-well dishes (~50,000 cells/well) or 6-well dishes (one million cells/well) coated with poly-L-ornithine (Sigma, P4957) in Neurobasal medium (Invitrogen) supplemented with 0.5 mM glutamine, 50 units/ml penicillin-streptomycin, 1% N2 and 2% B27 supplements (Invitrogen), and 1% non-dialyzed fetal bovine serum. Seven days after initial plating, the medium was supplemented with camptothecin (10 μ M). At appropriate times of culture, cells were fixed in methanol for 15 min and assessed for neuronal survival as described below. Alternatively, cells were collected and analyzed by Western blot analyses as described below.

Western Blot Analyses—Cortical neurons or differentiated stem cell cultures were dissociated and cultured as described above. Cortical neurons were washed twice in PBS and harvested in SDS-loading buffer (12). Differentiated stem cells were washed twice in PBS, harvested in IP buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 20 units/ml aprotinin, 10 mM β -glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate), and sonicated at 4 $^{\circ}$ C for 10 s. Samples containing 10 μ g of protein were loaded onto SDS-polyacrylamide gels and transferred onto nitrocellulose membrane as described previously (11). Blots were probed with anti-p53 1C12 (Cell Signaling Technology; 1:2000), calpain-cleaved spectrin derived from a calpain-cleaved spectrin epitope as reported previously (33), MDM2 (Santa Cruz Biotechnology; 1:1000), or anti- β -actin (Sigma; 1:3000) primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody (Bio-Rad; 1:3000) as indicated.

Immunohistochemistry—Neurons cultured as described above in 6-well dishes were untreated or treated with camptothecin with and without calpain inhibitor cotreatment. Neuronal cultures were fixed in 4% paraformaldehyde in phosphate buffer solution for 30 min at 4 $^{\circ}$ C as described previously (10, 15). The cells were incubated with anti-cyto-

chrome *c* (BD Biosciences; 1:400) or anti-p53 1C12 (Cell Signaling Technology; 1:2000) primary and CY-3 (Jackson ImmunoResearch Laboratories) secondary antibodies. To distinguish neuronal phenotypes from glia in differentiated stem cell cultures, we used anti- β -III tubulin primary (Berkeley Antibody; 1:500) and streptavidin Alexa Fluor (Molecular Probes 1:200) secondary antibodies. Finally, cultures were incubated with Hoechst 33258 (0.25 μ g/ml) for 10 min. Cells were visualized under fluorescent microscopy. The percentage of neuronal survival in stem cell cultures was obtained by whole well counting of shrunken and condensed nuclei of β -III tubulin-positive cells. Similarly, in cortical cultures infected with adenovirus expressing calpastatin and/or GFP, the percentage of shrunken nuclei and the expression of p53 and cytochrome *c* of infected cells were assessed by randomly counting a total of 150 cells/well. Each data point is the mean \pm S.E. from three cultures.

Caspase Activity—Cortical neurons were harvested at the indicated times for caspase activity. Briefly, cells were washed twice in PBS and collected in caspase lysis buffer as described previously (15). The cells were incubated on ice for 20 min and briefly sonicated for 3 s. The extracts were then centrifuged for 15 min at 12,000 rpm in an Eppendorf tabletop centrifuge. The supernatants were collected and assayed for protein concentration by Bradford reagent (Bio-Rad). Equal amounts of protein (5 μ g) were incubated with the caspase-3 substrate DEVD-AFC as described previously, and fluorescence was measured using a fluorometer (400 nm excitation, 505 nm emission), as described previously (38).

RESULTS

Activation of Calpains following DNA Damage—We first determined whether calpains are activated following DNA damage. Treatment of embryonic cortical neurons with the DNA topoisomerase-1 inhibitor, camptothecin, induces apoptotic death (6–10). We have shown previously that the commitment point of this death is ~6 h after initiation of camptothecin exposure (12). Accordingly, the death signals that mediate this commitment must occur prior to this time point. Therefore, we determined whether calpain activity occurred within this commitment window by analyzing for the presence of calpain-mediated proteolysis utilizing an epitope-specific antibody directed against the calpain cleavage site of α -spectrin. Antibodies raised against this epitope of α spectrin have been shown previously to be specific for calpain-cleaved spectrin and not for the proteolytic products of caspases (33). As shown in Fig. 1, calpain activity begins to increase ~2 h after camptothecin exposure and was robustly activated at 8 h. Consistent with this observation, cotreatment of neuronal cultures with the calpain inhibitor MDL 28170 blocked the induction of the calpain-cleaved spectrin signal. Increased calpain activity was not due to an increase in cellular amounts of calpains, because levels of the calpain μ and m large subunits remained relatively constant throughout the 8-h course of camptothecin exposure as determined by Western blot analyses (data not shown). This early induction of calpain activity is consistent with its role as a proximal death signal that may mediate death commitment.

Calpain Inhibition and DNA Damage-induced Death—We next determined the functional consequences of calpain activation following DNA damage-induced neuronal death. To examine this, we first utilized the pharmacological calpain inhibitors MDL 28170 (39) and PD 150606 (40). As shown in Fig. 2, A and B, both inhibitors blocked neuronal death with *in vivo* IC₅₀ values of ~50 and 25 μ M, respectively, at 14 h following camptothecin exposure. However, assessment of protection at later points could not be performed due to toxicity of the agents (data not shown). Neurons protected with the calpain inhibitors have well defined, healthy somas, whereas those treated with camptothecin alone show phase bright apoptotic bodies (Fig. 2C). Although neuritic processes were present in both PD 150606- and MDL 28170-treated cultures, they were generally fewer in number relative to untreated cells. These observations are consistent with reports linking calpains to process outgrowth

¹ The abbreviations used are: EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; GFP, green fluorescent protein; CDK, cyclin-dependent kinase; ATM, ataxia telangiectasia-mutated protein kinase; ATR, ATM and Rad-3-related protein kinase.

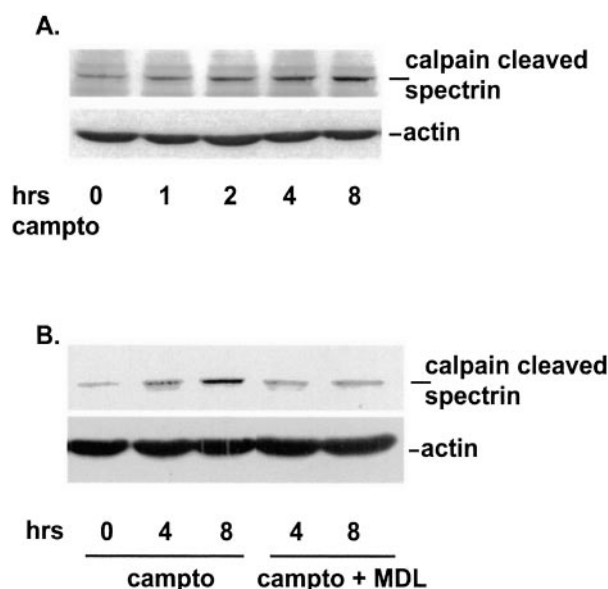


FIG. 1. Calpains are activated in cultured cortical neurons following DNA damage. Cortical cultures were treated with camptothecin (*campto*; 10 μM) with and without cotreatment with the calpain inhibitor MDL 28170 as indicated. Cell extracts were analyzed for calpain activity by Western blot analyses utilizing an epitope-specific antibody for calpain-cleaved spectrin. The same blots were then stripped and probed for actin as a loading control. *A*, time course of calpain activation. *B*, effect of the calpain inhibitor MDL 28170 (*campto* + *MDL*) on calpain activation.

(41). Although the protection offered by these inhibitors is concordant with the involvement of calpains in neuronal death, there are possible alternative explanations for these effects. Importantly, these inhibitors are known to block other targets, which include cathepsins (40) and calcineurins (43). These potentially confounding effects have made interpretation of pharmacological inhibitor experiments difficult. To overcome this, we explored two additional and more targeted ways of calpain inhibition.

In the first strategy, we infected cortical cells with an adenovirus expressing calpastatin, the endogenous calpain inhibitor. Cortical neuronal cultures were infected at the time of plating with recombinant adenovirus expressing either GFP and calpastatin or GFP alone. After 24 h, the cultures were exposed to camptothecin for 14 h. As shown in Fig. 3, neurons overexpressing calpastatin had healthy somas, intact nuclei, and reduced death when compared with GFP-expressing controls similarly treated with camptothecin (~90% survival with calpastatin *versus* 50% with GFP).

To provide further evidence for the involvement of calpain in cell death, we explored whether calpain deficient neurons were also resistant to DNA damage-induced death. Previous reports have indicated that cells deficient in the small calpain subunit (*Capn4*) lack both calpain μ and m activity (17). Because, *Capn4*^{-/-} mice do not survive beyond embryonic days 10–12 (17), it is not possible to obtain fully differentiated cortical neurons. To circumvent this problem, we isolated neuronal stem cells from the forebrain region of *Capn4*^{+/+}, *Capn4*^{+/-}, and *Capn4*^{-/-} embryonic day 10.5 embryos. Neuronal stem cells were then exposed to differentiation medium as described previously (37), treated with camptothecin, and fixed. Neuronally differentiated cells were identified by staining with the neuronal marker β -III tubulin, and death was assessed by nuclear Hoechst staining. As shown in Fig. 4, there was significantly more survival in calpain-deficient cells when compared with litter mate controls. Taken together, these data show that calpain inhibition by multiple means inhibits neuronal loss and

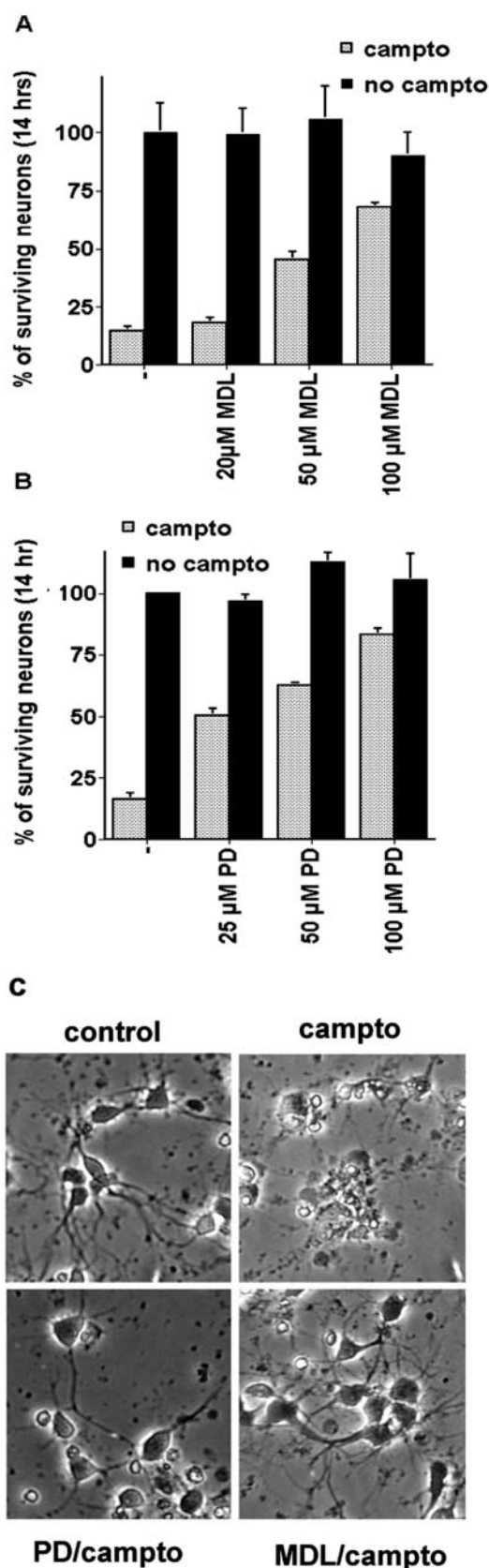


FIG. 2. Pharmacological calpain inhibitors block death of cortical neurons induced by DNA damage. Cortical cultures were treated with camptothecin (*campto*; 10 μM) with and without MDL 28170 (*A*) and PD 150606 (*B*) cotreatment at the concentrations indicated. Each point is the mean \pm S.E. of data from three cultures. *C*, phase contrast micrographs of cortical neurons with the indicated treatments. All images were obtained at 12 h post camptothecin treatment.

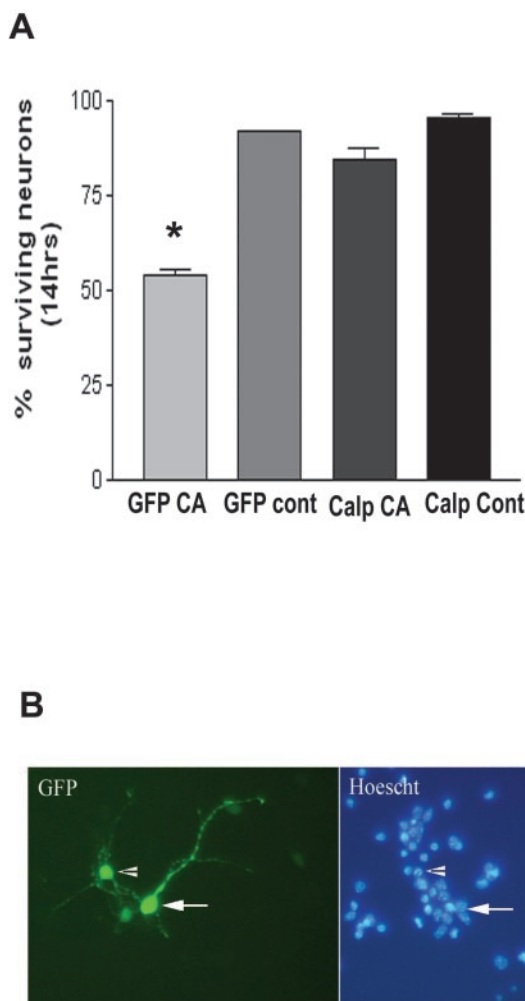


FIG. 3. Calpastatin expression inhibits death of cortical neurons by DNA damage. Cortical neurons were infected with adenoviruses expressing GFP and calpastatin or GFP alone and treated with camptothecin (CA; 10 μ M) for 14 h. *A*, each point is the mean \pm S.E. of data from three cultures. *Asterisk* denotes significance ($p < 0.001$). *B*, representative fluorescent image of GFP-positive neurons treated with camptothecin (14 h) and analyzed for Hoechst stain. *Arrowhead* represents a GFP-positive dead cell. *Arrow* represents a GFP-positive alive cell.

provide strong evidence for the importance of calpains in neuronal death evoked by DNA damage.

Calpains Mediate p53 Induction—We next examined the pathways by which calpains may mediate DNA damage mediated neuronal death. We (11) and others (14) have reported previously that p53 deficiency blocks death following camptothecin treatment. As shown in Fig. 5, cellular levels of p53 protein were elevated by 2 h and increased up to 8 h after camptothecin treatment. Little or no p53 was detectable in control cultures. We next asked whether calpains might modulate p53 induction. Interestingly, there was a reduction and a significant delay in p53 induction in cultures cotreated with the MDL 28170 inhibitor (Fig. 5, *A* and *C*). No p53 was detected at 4 h, and only \sim 50% of p53 levels was present at 8 h in comparison with neurons treated with camptothecin alone. Similar results were obtained with the PD 150606 inhibitor (Fig. 5*B*).

To confirm these results and rule out the possibility that inhibition of p53 was due to nonspecific effects of the pharmacological calpain inhibitors, we also examined the effects of calpastatin expression on p53 induction evoked by camptothecin. Neuronal cultures were infected with GFP and calpastatin or GFP-only adenovirus. Following camptothecin exposure,

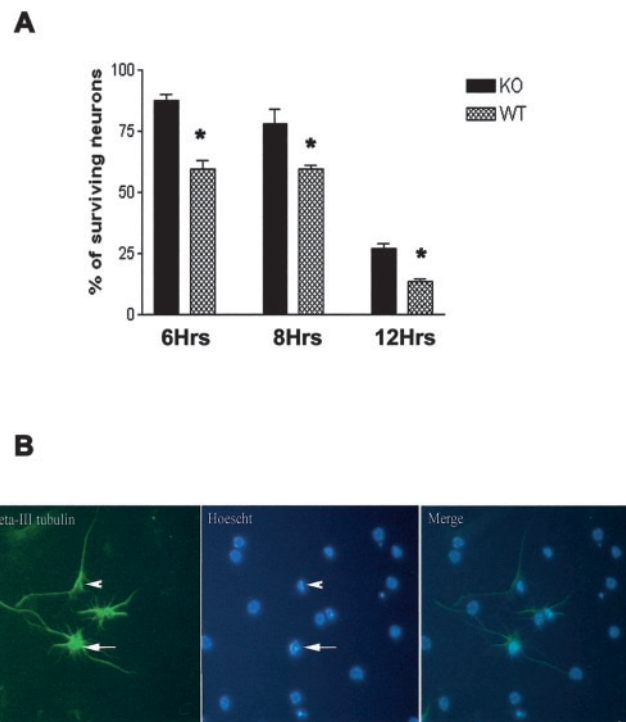


FIG. 4. *Capn4*^{-/-} neuronally differentiated cells are resistant to death induced by DNA damage. Cells were treated with camptothecin (10 μ M) for the indicated times. *A*, the percentage of surviving β -III tubulin positive neurons per well was assessed. Each point is the mean \pm S.E. of data from three cultures. *Asterisk* denotes significance ($p < 0.05$). *B*, representative fluorescent image of β -III tubulin-positive neuronally differentiated cells (green) treated with camptothecin (12 h) and analyzed for Hoechst stain (blue). *Arrowhead* represents β -III tubulin-positive dead cell. *Arrow* represents β -III tubulin live cell.

neurons were fixed, and GFP-positive neurons assessed for p53 induction by immunofluorescence. As shown in Fig. 6, 85% of GFP-expressing neurons were p53 positive. In contrast, only 6% of neurons expressing calpastatin were positive for p53. Consistent with these results, differentiated stem cell cultures from *Capn4*^{-/-} embryos also showed less p53 induction when compared with litter mate controls in response to camptothecin exposure (data not shown). Taken together, the pharmacological and molecular evidence indicates that p53 induction is mediated through calpain activation.

MDM2 has been implicated in the stability of p53 (see Ref. 44 for review). Because we determined that calpains regulate p53 induction, we next examined whether MDM2 levels were modulated during camptothecin-induced death. However, as shown in Fig. 7, MDM2 levels did not change appreciably following camptothecin treatment, and calpain inhibitor cotreatment did not affect expression of MDM2. These results indicate that calpains do not regulate p53 by modulating MDM2 protein levels.

Calpain Inhibition Blocks Activation of the Mitochondrial Death Signal—We have shown previously that p53 is required for activation of the mitochondrial pathway of death, which includes release of cytochrome *c* from mitochondria and caspase activation (10, 12, 15). Also in this paradigm, inhibition of caspases, either by general caspase inhibitors or caspase 3 deficiency, transiently protects neurons from camptothecin-induced apoptosis (10, 15). If calpains indeed act upstream of p53, one would anticipate that the mitochondrial pathway of death would also be inhibited. To test this expectation, we determined the effects of calpain inhibition on cytochrome *c* release and caspase 3-like activation. As we have shown previously in cortical neurons, cytochrome *c* is localized to punc-

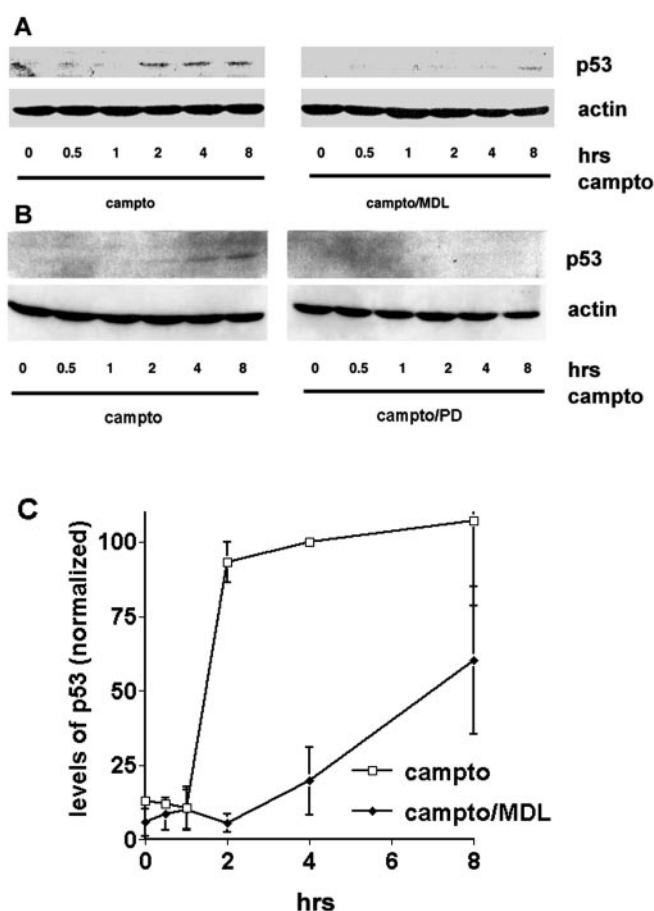


FIG. 5. Induction of p53 is inhibited by pharmacological calpain inhibitors. Cortical cultures were treated with camptothecin (*campto*; 10 μM) with and without MDL 28170 (100 μM) (A and C) and PD 150606 (50 μM) (B) for the times indicated. A and B, p53 levels were assessed by Western blot analyses, and representative blots are shown. C, densitometric analyses of p53 levels for cultures cotreated with MDL 28170 (100 μM). Each point is the mean \pm S.E. of data from three experiments and is normalized to a signal of p53 at 4 h of camptothecin treatment.

tate mitochondrial compartments when visualized by immunofluorescence (10, 15) (see also Fig. 8A). However when cortical cultures are treated with camptothecin for 12 h, cytochrome *c* staining is lost, and nuclei become condensed and fragmented. Cotreatment with the MDL28170 calpain inhibitor prevented both the loss of cytochrome *c* staining and the nuclear fragmentation associated with apoptosis (Fig. 8). Similar results are obtained with the PD150606 inhibitor. However, in this case, the nuclear morphology was not completely normal and displayed a slightly more rounded appearance. This may reflect the change in general cellular morphology and neuritic retraction, which is more evident with PD 150606. Nevertheless, cytochrome *c* staining was still present in these neurons. Cytochrome *c*-positive neurons are quantified in Fig. 8B. As indicated, $\sim 85\%$ of neurons in control cultures were positive for cytochrome *c* versus $\sim 35\%$ in camptothecin-treated cultures. Importantly, cotreatment with the MDL or PD compounds resulted in an increased number of cytochrome *c* positive neurons ($\sim 65\text{--}75\%$).

In accordance with these results, we also determined that calpastatin expression inhibited cytochrome *c* release in response to camptothecin. Cultures were infected with recombinant adenovirus expressing either GFP or GFP-calpastatin. As shown in Fig. 8C, only 19% of neurons expressing GFP control and treated with camptothecin were positive for cytochrome *c*.

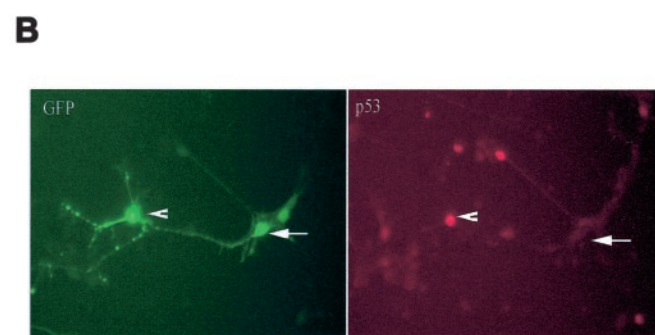
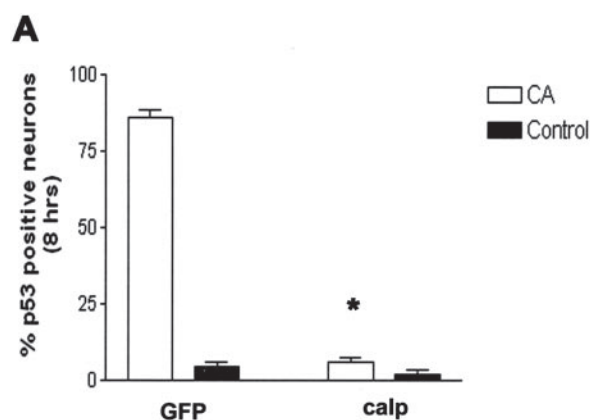


FIG. 6. Calpastatin expression and/or calpain deficiency blocks p53 induction evoked by camptothecin. A, percentage of cells expressing GFP and calpastatin or GFP only that are positive for p53 after 8 h of camptothecin incubation. Each point is the mean \pm S.E. of data from three cultures. Asterisk denotes significance ($p < 0.0001$). B, representative fluorescent image of p53-positive neurons expressing calpastatin after 8 h of camptothecin exposure. Arrowhead represents a GFP- and a p53-negative cell. Arrow represents a GFP-positive, p53-positive cell.

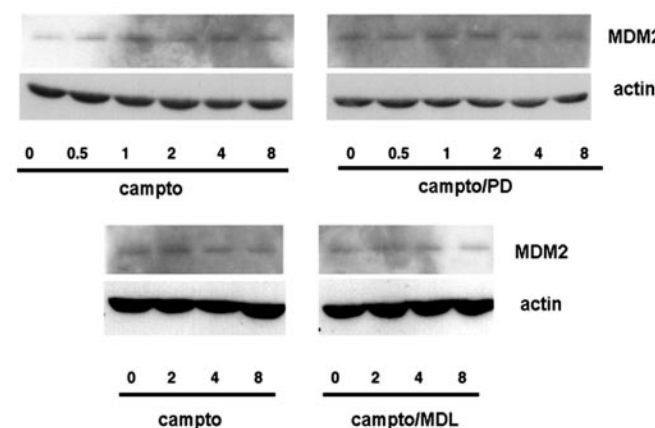


FIG. 7. Calpain inhibition does not affect MDM2 levels. Cortical cultures were treated with camptothecin (*campto*; 10 μM) with and without PD 150606 (50 μM) or MDL 28170 (100 μM) for the times indicated. MDM2 levels were assessed by Western blot analyses.

In contrast 73% of neurons expressing calpastatin showed intact cytochrome *c* labeling.

Cytochrome *c* release is required for activation of the apoptosome complex and subsequent downstream effector caspases such as caspase 3 (45). Accordingly, we would predict that caspase 3-like activity should also be inhibited with calpain inhibition. Consistent with this notion, cotreatment of cultures with either calpain inhibitor significantly inhibited the activa-

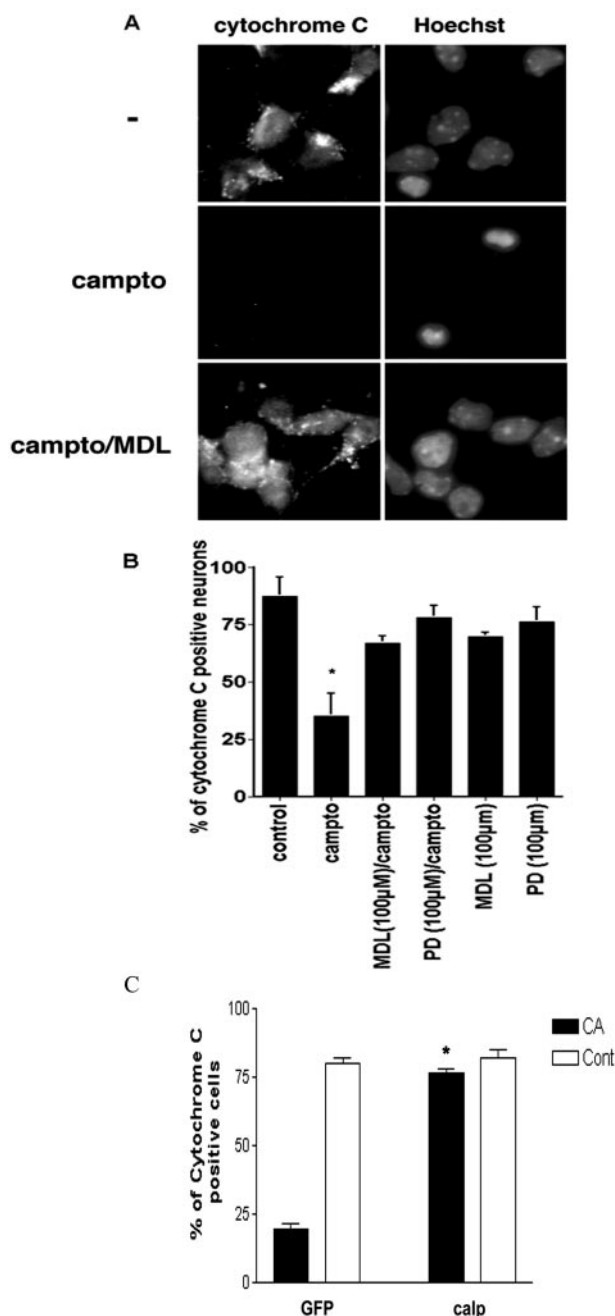


FIG. 8. Calpain inhibitors and calpastatin expression blocks loss of cytochrome *c* from cortical cultures treated with camptothecin (*campto*). A, fluorescent images of camptothecin-treated cortical cultures cotreated with or without MDL 28170 (100 μ M) as indicated and analyzed for cytochrome *c* or Hoechst stain. B, quantitation of cytochrome *c* positive cells with the treatments as indicated. Each point is the mean \pm S.E. of data from three cultures. Asterisk denotes significance ($p < 0.05$). C, percentage of cytochrome *c* positive cells expressing calpastatin (*calp*) with 10 h treatment of camptothecin. Each point is the mean \pm S.E. of data from three cultures. Asterisk denotes significance ($p < 0.0001$).

tion of caspase 3-like activity by $\sim 70\%$ as measured by DEVD-AFC cleavage activity (Fig. 9). Taken together, these findings indicate that calpain proteolysis mediates the up-regulation of p53 and consequent events, including the release of cytochrome *c* and the activation of caspases, that result from DNA damage.

DISCUSSION

DNA damage is an important initiator of neuronal apoptosis. Radiation, cancer chemotherapeutic agents, ischemic insult,

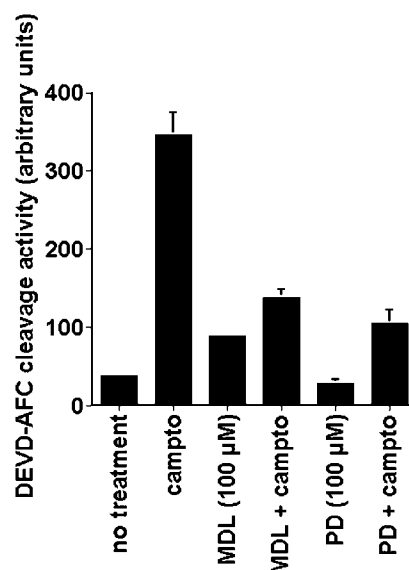


FIG. 9. Calpain inhibition blocks activation of caspase 3-like activity following DNA damage. Cortical neuronal cultures were treated with camptothecin with and without MDL 28170 (100 μ M) or PD 150606 (100 μ M) cotreatment. DEVD-AFC cleavage activity was determined as described under "Experimental Procedures." Each point is the mean \pm S.E. of data from three cultures.

and oxidative stress all induce significant levels of DNA damage and neuronal death both *in vitro* and *in vivo* (6–9, 46–53). An important and required component of DNA damage induced death is the tumor suppressor p53. For example, p53 is up-regulated following camptothecin treatment (11, 54), irradiation (55), and ischemic insult (56). Moreover, deficiency of p53 has been shown to protect against neuronal death in all three paradigms (11, 54, 55, 57). However, the signal(s) which impact on p53 stability/activity are not completely clear.

The role of calpains in neuronal death has been suggested but remains controversial. In addition, the manner by which calpains mediate neuronal death evoked by DNA damage is also unclear. In this paper, we provide multiple lines of molecular and pharmacological evidence that calpains do participate in neuronal death induced by the DNA-damaging agent camptothecin and, furthermore, that they act to regulate the p53 signaling axis.

Calpain Requirement following DNA Damage—As evidenced by the accumulation of calpain-cleaved spectrin, induction of calpain activity is an early event that precedes the commitment point of death, which we have previously established to be ~ 6 h following camptothecin exposure (12). This observation suggests that calpain activation is an upstream mediator of death in this model. Supporting this hypothesis, we show that two distinct pharmacological inhibitors of calpains block the death of neurons exposed to camptothecin. Although these results support the proposed role of calpain-mediated death signals in this death model, we could not exclude the possibility that these calpain inhibitors may be affecting other signals. In this regard, MDL-28170 and PD inhibitors have been reported to inhibit cathepsins (43) and calcineurins (40) respectively, although at higher concentrations. This is important, because other properties associated with pharmacological calpain inhibition, such as regulation of cell division, have not been observed in calpain-deficient cells, suggesting that calpain inhibitors may act through alternative mechanisms (17). Accordingly, we examined two additional molecular means of calpain inhibition. In this regard, our observation that calpastatin expression as well as calpain deficiency significantly inhibit death indicates that calpains do participate in the DNA damage-

induced death signal caused by camptothecin. Interestingly, neuronally differentiated calpain-deficient cells displayed less resistance than that observed in cortical neurons expressing calpastatin or treated with pharmacological inhibitors. This may be due to differences in the calpain involvement between neuronally differentiated stem cells and cortical neurons obtained from embryonic day 15 mice. Importantly, it is clear from all the calpain functional data presented that this protease participates in, but does not solely regulate, DNA damage-induced neuronal death (see below for further discussion). Finally, it must be noted that the present study examines DNA damage evoked only by camptothecin. Participation of calpains in other forms of DNA damage will have to be determined empirically.

Calpains and Regulation of p53—How do calpains regulate DNA damage-induced neuronal death? Our results indicate that this occurs through the p53 signaling axis. p53 levels are elevated early and prior to death commitment, and p53 deficiency prevents neuronal death evoked by DNA damage (11, 54). p53 is also an absolute requirement for activation of the distal death effector pathway (Bax translocation, cytochrome *c* release, and caspase activation) (10, 15, 54). We show that calpain inhibition by pharmacological agents, knockouts, or calpastatin overexpression significantly blocked the activation of p53 following camptothecin treatment. If calpains act upstream of p53, one prediction would be that calpain inhibition would also block the events downstream of p53 activation. Consistent with this idea, the inhibition of calpain activity by calpastatin expression as well as pharmacological inhibition prevented the release of cytochrome *c* and the activation of caspases.

Although it is unclear how calpain activation modulates p53 levels, it is unlikely that it has a direct effect on p53 stability. First, MDM2, a regulator of p53 levels, does not change appreciably during camptothecin treatment. This observation rules out the possibility that calpain cleavage of MDM2 accounts for the elevation in p53. Secondly, and in contrast with our observations, p53 has been proposed to be a substrate of calpains under certain conditions (58, 59). Calpain inhibitors have been shown to up-regulate p53 levels and lead to cell cycle arrest and apoptosis in proliferating cell systems (60, 61). These effects are opposite to what is reported here. These differences may be due to the cellular context of proliferating cells *versus* neuronal systems. Interestingly, calpains are known to cleave I κ B, a negative regulator of the NF κ B pathway (62, 63). NF κ B, in turn, can activate p53 through direct transcriptional means (64). We have shown that NF κ B is activated by DNA damage and also regulates p53 activation.² Therefore, an attractive hypothesis is that calpain-mediated activation of the NF κ B pathway may, in turn, regulate p53. However, validation of this model will require further study.

The transient nature of p53 suppression by calpain inhibition also suggests that there may be alternative/multiple mechanisms by which p53 stability is regulated in neurons. Although p53 regulation is complex, three pathways relevant in this death paradigm will be considered. First, cyclin-dependent kinases (CDKs) can directly phosphorylate p53, resulting in its enhanced stability (65). This report is interesting, because we have previously implicated CDKs as activated and required for death (7–9). Secondly, p19 ARF has been implicated in mediating oncogenic up-regulation of p53. In this case, deregulated E2F1 has been shown to up-regulate p19ARF, which then inhibits the ability of MDM2 to degrade p53 (66). Interestingly, E2F family members have also been implicated in campto-

thecin-induced death. For example, expression of dominant negative DP1, an obligate binding partner to E2F members, inhibits death in this paradigm (9). These observations raise the possibility that CDK activation regulates p53 either directly or through the Rb/E2F1/ARF19 pathway. However, our observation that inhibition of CDKs has no effect on p53 levels makes these two possibilities unlikely (12). The third pathway of p53 stability involves the phosphatidylinositol 3-kinase-like ATM/ATR family of kinases. Previous reports indicated that these kinases phosphorylate p53 directly on Ser-15 or indirectly on Ser-20 through activation of Chk2 (42, 67–71). In this regard, our results indicate that ATM but not Chk2 also modulates the stability of p53 and consequent death.² Therefore, it is likely that multiple signals relating to calpains, NF κ B, and ATM regulate p53 activation, and it will be important to explore how these signals ultimately coordinate the p53 pathway. Taken together, our observations suggest that calpain-mediated events regulate p53 activity and provide one explanation of how calpain inhibition is an effective neuro-protectant in p53-mediated death paradigms.

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