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Ceramide triggers an NF-κB-dependent survival pathway through calpain

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Abstract

We have shown that C2 ceramide, a cell-permeable analog of this lipid second messenger, triggers an NF-κB dependent survival pathway that counteracts cell death. Activation of NF-κB and subsequent induction of prosurvival genes relies on calpain activity and is prevented on silencing of the calpain small subunit (Capn4) that is required for the function of ubiquitous calpains. We have demonstrated that p105 (NF-κB1) and its proteolytic product p50 can be targets of micro- and milli-calpain *in vitro* and that a p50 deletion mutant, lacking both the N- and the C-terminal ends, is resistant to calpain-mediated degradation. Capn4 silencing results in stabilization of endogenous p105 and p50 in diverse human cell lines. Furthermore, p105 processing and activation of NF-κB survival genes in response to C2 ceramide is impaired in Capn4−/− mouse embryonic fibroblasts defective in calpain activity. Altogether, these data argue for the existence of a ceramide–calpain–NF-κB axis with prosurvival functions.
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Keywords: calpain; NF-κB; ceramide; apoptosis

Abbreviations: TNF-α, tumor necrosis factor-alpha; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LUC, luciferase; MEFs, mouse embryonic fibroblasts; PARP, poly(ADP-ribose)polymerase; siRNA, small interfering RNA

Introduction

Natural ceramides are potent lipid second messengers produced by the action of sphingomyelinases that catalyze the hydrolysis of sphingomyelin to form ceramide and phosphocholine. They are thought to be involved in mediating the effect of various cytokines including tumor necrosis factor alpha (TNF-α) and interferon-γ, Fas ligand, ionizing radiation and anticancer drugs such as adriamicin, etoposide and camptothecin, with different outcomes including growth arrest, differentiation, apoptosis, autophagy or necrosis, depending on the cellular context; however, the involved molecular mechanisms are still ill defined.[1]

Cell-permeable ceramide analogs have been widely used to mimic the action of endogenous ceramides and were reported to exert their biological effect directly as a consequence of Ca^{2+} release from intracellular stores,[2] but also through activation of Raf-1, mitogen-activated protein/extracellular regulated kinase (ERK) kinase (MEK) and ERKs.[3] In particular, ceramide activates ceramide-activated protein (CAP) kinase that in turn phosphorylates Raf-1 on Thr 269, increasing its activity towards MEK (MAP kinase or ERK kinase).[4] Moreover, C2 ceramide selectively modulates calpain I expression levels, thus decreasing tau levels in differentiated PC12 cells.[5]

The final outcome of C2 ceramide treatment changes according to its concentration and the cellular context. C2 ceramide was shown to trigger growth arrest in a number of cell types,[6–8] and different types of cell death such as apoptosis,[9–11] necrosis[12,13] and autophagocytosis.[14] All these findings make ceramide appealing for cancer treatment, notably a Phase II clinical trial to study the effectiveness of ceramide cream in treating women who have cutaneus breast cancer is going on (sponsored by the National Cancer Institute). Given the potential importance of ceramide as an anticancer drug, it is fundamental to investigate whether it can activate any parallel survival pathway, as reported for other drugs. This will help to design combined and appropriate therapies for specific molecular defects. In support of the hypothesis that ceramide could play a role as inducer of survival pathways, C2 ceramide was shown to upregulate the expression of antiapoptotic genes such as *Bcl-2* and *Bcl-w* in HC11 mammary epithelial cells[15] and cyclooxygenase (COX-2) in mouse macrophages.[16] Moreover, C2 ceramide-induced expressions of inducible nitric oxide synthase (iNos) in rat primary astrocytes[17] and in rat vascular smooth muscle cells[18] were reported to occur via NF-κB activation. The activation of the transcription factor NF-κB by other apoptotis-inducing stimuli such as TNF-α, ionizing radiation or certain chemotherapeutic compounds (e.g. doxorubicin) was previously described and has been found to protect cells from apoptosis. Indeed when NF-κB is inhibited, apoptotic killing by these reagents is enhanced.[19] The NF-κB/REL family of transcription factors pivotally control the inflammatory and immune response, as well as other genetic programs that are central to cell growth and survival. The cytoplasmic regulation of NF-κB activation involves stimulus-coupled phosphorylation, ubiquitination and proteasome-mediated degradation of the i-κB family members, resulting in the liberation of the NF-κB heterodimer and its rapid translocation into the nucleus.[20,21] A number of recent reports indicate that

cytoplasmic activation of the transcription factor NF-κB involves the inducible calpain/calpastatin system, besides the constitutive proteasome pathway. In particular, calpain 3 deficiency is associated with myonuclear apoptosis and profound perturbation of the i-κBα/NF-κB pathway in limb-girdle muscular dystrophy type 2A.[22] In addition, TNF-α activates cytosolic calpains to degrade the NF-κB inhibitor i-κBα.[23] Interestingly, data from different laboratories point to a link between ceramide, calpains and NF-κB activation. In particular, Kouba *et al*.[24] showed that C2 ceramide activates NF-κB in dermal fibroblasts and that calpain inhibitors can prevent such activation, arguing for the involvement of calpain in NF-κB induction by ceramide.

Calpains constitute a large family of cytosolic calcium-dependent proteases which play an important role in the regulation of intracellular activities including cell spreading and migration,[25,26] myoblast fusion, programmed cell death and cell cycle progression.[27] The ubiquitous calpains, Calpain I (micro-calpain) and II (milli-calpain), consist of a large 80 kDa subunit encoded by the *Capn1* and *Capn2* genes, respectively, and a common 28 kDa small subunit encoded by *Capn4*. Calpains are activated by calcium and negatively regulated by their specific endogenous inhibitor calpastatin. Binding of ligand to EGFR was shown to lead to activation of milli-calpain subsequent to ERK/MAP kinase signalling.[28,29] Endoplasmic reticulum (ER) stress-inducing agents such as thapsigargin, ionomycin and ceramides are widely used as triggers of calpain activity[27] to elucidate the molecular events underlying calpain-involved signalling.

Calpain-mediated cleavage regulates the activity of a number of diverse substrates, including cytoskeletal proteins such as spectrin and talin, kinases such as PKC, apoptotic proteins such as caspases and bax, transcription factors such as Fos and Jun, as well as the NF-κB inhibitor iκ-Bα.[27] In the present work, we have demonstrated that ceramide triggers an NF-κB-dependent survival pathway that counteracts ceramide-induced cell death. Such activation relies on ubiquitous calpain function and cleavage of the NF-κB inhibitor p105 (NF-κB1).

Results

Cell death induced by C2 ceramide is enhanced in p65−/− mouse embryonic fibroblasts (MEFs)

The cell-permeable analog C2 ceramide has been extensively used to study ceramide-dependent signalling in tissue culture. A large body of data strongly support that C2 ceramide can trigger cell death of various kinds, including apoptosis, necrosis and autophagocytosis.[30] On the other hand, other studies show that C2 ceramide can switch a NF-κB-dependent survival pathway on. We hypothesized that both divergent pathways were triggered by C2 ceramide and, in particular, that the NF-κB pathway could function as a lifebuoy to prevent death induction. A collection of cell lines was utilized to monitor cell death after treatment with 50 and 100 μM C2 ceramide for 8 h. Cell death was quantified by means of a colorimetric kit that allows one to measure lactate dehydrogenase (LDH) release in the medium, as an indicator of cytoplasmic membrane damage. Both dihydroceramide C2, an inactive form of C2 ceramide, and solvent alone did not have any effect on cell viability, indicating the specificity of the response to C2 ceramide. Treatment with solvent alone was utilized as a baseline control for all the *in vivo* experiments. The graph shown in Figure 1a illustrates the increase in cell death of C2 ceramide-treated cells with respect to control cells treated with solvent alone. C2 ceramide was cytotoxic for each cell line analyzed and its toxicity sharply increased as a function of concentration. To investigate any role played by NF-κB in modulating C2 ceramide-triggered cell death, we performed the same assay in MEFs derived from p50 and p65 knockout mice. Wild-type MEFs were included as a control. The cells were incubated with 50 or 100 μM C2 ceramide or solvent as negative control for 16 h. Afterwards, the amount of LDH released in the medium was measured to determine cell death. The data in Figure 1b illustrate the increase in cell death occurring after C2 ceramide incubation in comparison to the basal level detected after incubating the cells with solvent alone. Cell death appears to be higher in p50 lacking fibroblasts with respect to control wild-type MEFs and even higher in p65−/− MEFs, indicating that knocking down NF-κB activity significantly potentiates cell death. The differential death response to ceramide is maintained also after longer

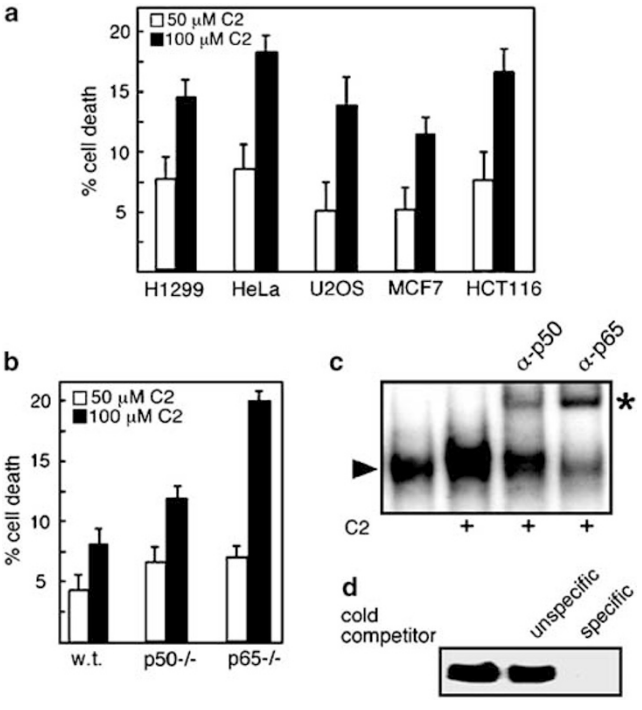

Figure 1 C2 ceramide induces both cell death and NF-κB activation. Panel **a**: H1299, HeLa, U2OS, MCF7 and HCT116 cells were incubated with 50 or 100 μM C2 ceramide or solvent alone for 8 h. Afterwards, LDH released in the medium was quantified using a commercial kit. The values reported in the graph represent the increase in cell death occurring in ceramide-treated cells compared to control cells incubated with solvent alone. Panel **b**: Wild-type MEFs, p50−/− and p65−/− MEFs were incubated with 50 or 100 μM C2 ceramide or solvent alone for 16 h and cell death was quantified as described above. Panel **c**: Gel shift assay for NF-κB activation in NIH-3T3 cells treated with C2 ceramide for 30 min (as indicated by the + symbol) or solvent alone as negative control. In the indicated lanes, the induced complex was supershifted with anti-p50 or anti-p65 antibodies. Panel **d**: Competition experiments with 200- fold excess of specific and unspecific cold competitor as indicated

incubation times (data not shown), indicating that NF-κB is activating a true survival pathway.

To verify that C2 ceramide activated NF-κB, 3T3 cells were incubated with 100 μM C2 ceramide or solvent as negative control for 30 min. Nuclear extracts were then prepared and incubated with a ^{32}P-labelled NF-κB consensus oligonucleotide to monitor NF-κB-binding activity. Figure 1c shows that C2 ceramide treatment correlates with an increase in NF-κB-binding activity (indicated by an arrow) that could be supershifted (marked by an asterisk) by preincubating the reaction mixture with antibodies to either p50 or p65, and efficiently competed by a 200-fold excess of specific cold competitor.

Capn4 silencing correlates with an increase in cell death in response to C2 ceramide

The data reported above indicated that C2 ceramide (100 μM) is cytotoxic for a number of cell lines and cell death is even more severe in the absence of NF-κB. Independent studies have shown that C2 ceramide can activate calpain[5] and NF-κB,[31] or both.[24] In addition, calpain inhibitors can prevent NF-κB induction triggered by ceramide in dermal fibroblasts.[24] Interestingly, calpain has emerged as a parallel system for NF-κB activation. To further investigate the existence of a ceramide–calpain–NF-κB axis in human cells, we decided to assess the effect of Capn4 silencing on C2 ceramide–induced cell death. An effective reduction of Capn4 protein levels was evident 48 h after transfection of Capn4-specific small interfering RNA (siRNA) in H1299, HeLa and U2OS cells, whereas a nonfunctional control siRNA was ineffective (Figure 2a). Silenced cells were either treated with 100 μM C2 ceramide for 6 h, or solvent as negative control, and LDH release was quantified. Eight replicates of each experimental point were performed for each cytotoxicity assay. This assay was repeated at least three times for all the cell lines analyzed with similar outcomes. Figure 2b shows the increase in cell death measured in a typical experiment, which clearly indicates that Capn4 silencing amplifies the cell death response induced by C2 ceramide in all the cell lines tested. The highest increase was observed for HeLa cells. Analogous results were obtained by counting dead cells by trypan blue exclusion (data not shown). It must be noted that LDH release quantitation and trypan blue exclusion assays do not allow us to determine which type of death is switched on by C2 ceramide treatment; hence, the relative contribution of different mechanisms of cell death cannot be assessed in our experimental settings. Notably, Capn4 silencing *per se* induces a low, but reproducible increase in cell death.

To further investigate the effect of Capn4 silencing on the amplification of the cytotoxic effects induced by ceramide, we performed annexin V staining followed by FACS analysis. Annexin V exhibits antiphospholipase activity and binds to phosphatidylserine. Cells undergoing apoptosis acquire Annexin V-binding sites during apoptosis and provide a convenient method for detection of cells undergoing apoptosis. HeLa cells were transfected with Capn4 siRNA or a nonfunctional control siRNA. After 48 h, silenced cells were either treated with 50 μM C2 ceramide for 16 h, or solvent alone as negative control, and then stained with fluorescein-conjugated annexin V and analyzed by FACS. Figure 2c shows a typical FACS profile. The experiment was repeated three times with similar outcomes.

We obtained similar results by propidium iodide staining and FACS analysis to detect the sub-G1 population corresponding to apoptotic cells (not shown).

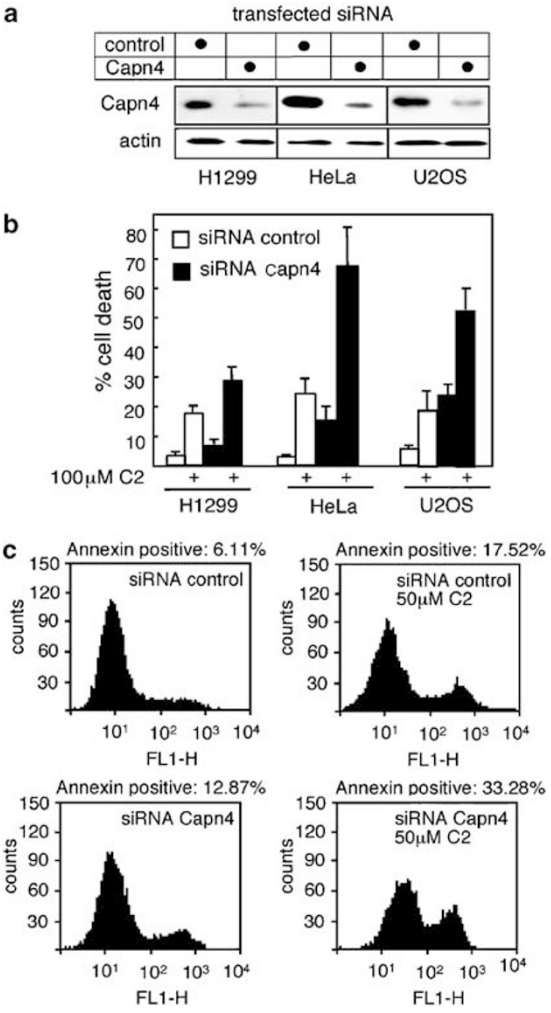

Figure 2 Capn4 silencing determines an increase in C2 ceramide-triggered cell death. Panel **a**: Capn4 siRNA, or control siRNA were transfected into H1299, HeLa or U2OS cells, as indicated. After 48 h, lysates were prepared and analyzed by Western blotting with antibodies to Capn4 or actin, as indicated. Panel **b**: H1299, HeLa and U2OS cells were transfected with Capn4-specific siRNA or control siRNA and 48 h later the cells were incubated with C2 ceramide or solvent alone for 6 h, cell death was measured with a colorimetric commercial kit. Eight replicates for each experimental condition were performed in each assay. The values reported in the graph represent the increase in cell death occurring in ceramide-treated cells compared to control cells incubated with solvent. The graph represents the mean of the eight replicates of a typical experiment. Panel **c**: HeLa cells were transfected with Capn4-specific siRNA or control siRNA and 48 h later the cells were incubated with 50 μM C2 ceramide or solvent alone for 16 h, afterwards cell death was measured by annexin V-FITC staining, followed by flow-cytometric analysis. Results of a representative experiment are shown, total percentage of annexin V-positive cells is shown at the top of each diagram

Activation of NF-κB by C2 ceramide is prevented upon calpain small subunit silencing

To directly study the role of calpain in NF-κB modulation, we addressed the effect of siRNA-based silencing of Capn4 on NF-κB activation in HeLa cells, where we detected the sharpest increase in C2 ceramide-induced cell death. HeLa cells were transfected with a firefly luciferase (LUC) reporter plasmid containing two κB sites together with Capn4-specific siRNA or control scrambled siRNA. A Renilla LUC reporter plasmid was included to normalize transfection efficiency. At 48 h after transfection, ceramide was added to the cells and LUC activity was monitored 4 and 8 h later. As shown in Figure 3a, κB-driven promoter strength is clearly enhanced upon C2 ceramide treatment in control cells, while the induction is significantly impaired in Capn4-silenced cells. To further test the involvement of calpain in NF-κB regulation following C2 ceramide treatment, we transiently silenced the endogenous calpain small subunit in HeLa cells to determine if a functional calpain was required for NF-κB induction. Ceramide was added to the silenced cells, and nuclear extracts were analyzed by gel retardation assays. As shown in Figure 3b, Capn4 silencing completely abolished C2 ceramide-triggered induction of NF-κB-binding activity. NF-κB activation by ceramide in control cells is coupled to a transient decrease in p105 protein levels (data not shown) as previously shown by others.[31]

We have shown that the activation of a κB-LUC reporter by ceramide is prevented in Capn4-silenced cells (Figure 3a). To further explore whether calpain-mediated NF-κB activation in response to C2 ceramide had any direct prosurvival role, we monitored promoter activity of *bcl-x*, a prosurvival NF-κB-activated gene. Transient transfections of a bcl-x-LUC construct were performed in Capn4 silenced and control HeLa cells, and LUC activity was measured. As shown in Figure 3c, the bcl-x promoter is activated in response to C2 ceramide. In Capn4-silenced HeLa cells, the basal bcl-x promoter activity is lower in comparison with control cells, and no increase is observed after C2 ceramide incubation. These observations lead us to speculate that NF-κB activation plays a role in attenuating C2 ceramide-induced cell death.

We next examined the expression of two prosurvival NF-κB-activated genes, iNOS and bclx-$_L$, in control- or Capn4-silenced cells that had been incubated in the presence of ceramide or solvent as negative control. As shown in Figure 3d, the protein levels of *iNOS* and *bclx-$_L$* increase after C2 ceramide treatment, and the increase is prevented by Capn4 silencing. As a first approach to gain insight into the molecular mechanisms underlying the observed increase in cell death, we examined the cleavage of the effecter caspase substrate, poly(ADP-ribose)polymerase (PARP). The increased C2 ceramide-induced cell death coupled to Capn4 silencing is clearly marked by an increase in the p85 PARP cleavage product (Figure 3d). Interestingly, the induction of PARP cleavage occurring in Capn4-silenced HeLa cells after ceramide treatment can be prevented by ectopic overexpression of the NF-κB-activated, antiapoptotic gene bcl-2, as shown in Figure 3e.

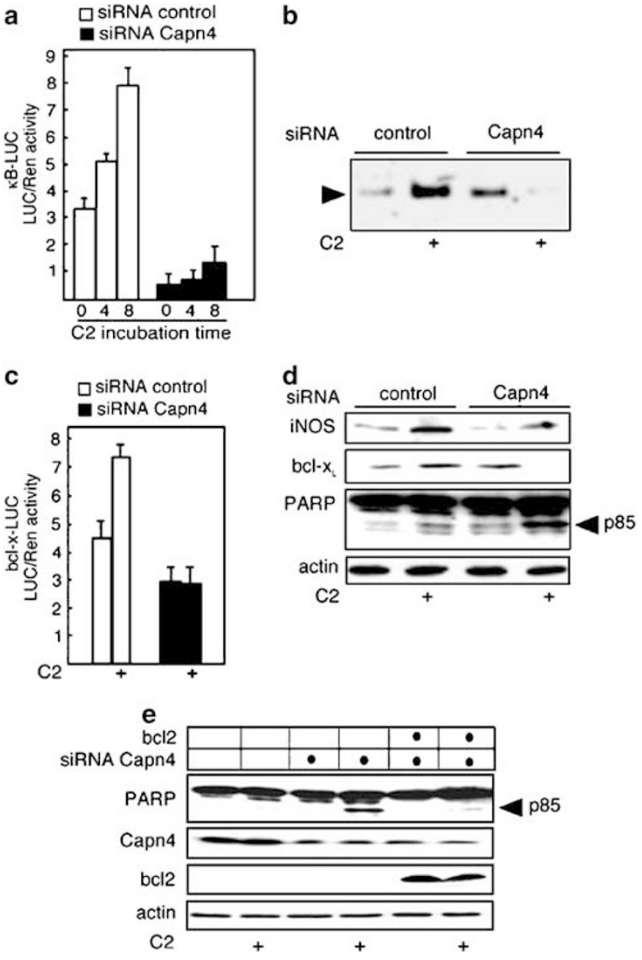

Figure 3 Capn4 silencing prevents activation of NF-κB-binding activity and function induced by C2 ceramide. Panel **a**: HeLa cells were transfected with a combination of control siRNA- or Capn4-specific siRNA and a LUC-expressing plasmid driven by an artificial promoter containing two κB sites. A Renilla expression (REN) vector was co-transfected for normalization. After 48 h, C2 ceramide or solvent alone was added to the cells. After 4 and 8 h, extracts were prepared and LUC/REN activity was measured. Panel **b**: HeLa cells were silenced with a control siRNA-or a Capn4-specific siRNA. After 48 h,the cells were induced for 30 min with ceramide or solvent alone and the nuclear extracts utilized to analyze NF-κB activation by means of gel shift. Panel **c**: HeLa cells were transfected with a combination of control siRNA or Capn4-specific siRNA- and a LUC-expressing plasmid driven by human bcl-x promoter. Renilla expression (REN) vector was co-transfected for normalization. After 48 h, C2 ceramide or solvent alone was added to the cells and 6 h later extracts were prepared and LUC/REN activity was measured. Panel **d**: The lysates of HeLa cells treated as indicated above were analyzed by immunoblotting to detect iNOS, bcl-x$_L$ and PARP. Actin levels were analyzed for standardization. The arrow marks p85, caspase-cleaved PARP. Panel **e**: HeLa cells were transfected with a combination of control siRNA- or Capn4-specific siRNA and a bcl-2 expression plasmid as indicated on the top of the figure. Empty vector was used as negative control. After 48 h, C2 ceramide or solvent alone was added to the cells and 6 h later extracts were prepared and analyzed by immunoblotting to detect endogenous PARP, overexpressed bcl-2 and actin as loading control

p105 and p50 can be cleaved by micro- and milli-calpain *in vitro*

Previous studies have shown that C2 ceramide activation of NF-κB does not involve the classical pathway of i-κB degradation in HL60 cells,[31] and it might rely on calpain

activity in dermal fibroblasts.[24] Polypeptide sequences enriched in proline (P), glutamate (E), serine (S) and threonine (T), known as PEST sequences, have been shown to mark proteins for recognition and destruction. In particular, the PEST domain of three proteins: beta-amyloid,[32] ATP-binding cassette transporter A1 (ABCA1)[33] and i-κB[34] have been reported to be involved in degradation by calpain.

A number of reports demonstrate the activation of the prototypical p50/p65 dimer among the other NF-κB family members as a consequence of p105 degradation.[35–37] P105 contains a PEST domain at its carboxyl-terminus;[38] therefore, we analyzed the susceptibility of p105 and its processing product p50 to proteolytic degradation by micro- and milli-calpain *in vitro*. I-κBα and gas2 proteins were used as positive and negative controls, respectively. All the proteins were produced and labelled with ^{35}S-methionine by *in vitro* transcription and translation using a reticulocyte-based commercial kit. Each protein product was used for *in vitro* protease assays with micro- and milli-calpain for 0, 5, 15 or 30 min, as indicated in Figure 4. As shown in Figure 4a and 4b, both micro- and milli-calpain can efficiently cleave p105 and p50. Although the micro- and milli-calpain cleavage patterns of p105 are slightly different, a residual undigested band of 36 kDa apparent molecular weight (marked by an arrow) appears to remain stable after proteolysis by both proteases.

A p50 deletion mutant corresponding to the Rel-homology domain is resistant to calpain degradation

In order to identify the regions of p105 and p50 involved in calpain degradation, we challenged a collection of deletion mutants schematized in Figure 5a by *in vitro* protease assays. The results obtained with micro-calpain are shown in Figure 5, panels b and c; similar results were obtained with milli-calpain (data not shown). The C-terminal deletion mutants were obtained by immunoprecipitation after transient transfection of the respective expression constructs with a GFP tag at the C-terminus in Phoenix cells. Each immunoprecipitation product was incubated for 20 min with calpain or buffer alone and then separated on SDS-PAGE, blotted onto nitrocellulose and detected with a GFP-specific antibody (Figure 5b). All the p105-derived constructs lacking increasing amounts of the C-terminal PEST sequences are efficiently degraded by calpain *in vitro*, demonstrating that the last 73 amino acids of p105 are not absolutely required for p105 degradation by calpain. To evaluate the involvement of the N-terminal region of p105, we analyzed a deletion mutant lacking the first 245 amino acids of p105, and I-κBγ, which is the product of a separate gene that lacks the first N-terminal 365 amino acids of p105.[39] These proteins were isolated by immunoprecipitation after transfection of N-terminus HA-tagged expression plasmids in Phoenix cells. Both the artificial construct and I-κBγ were efficiently digested in the *in vitro* assay (Figure 5b).

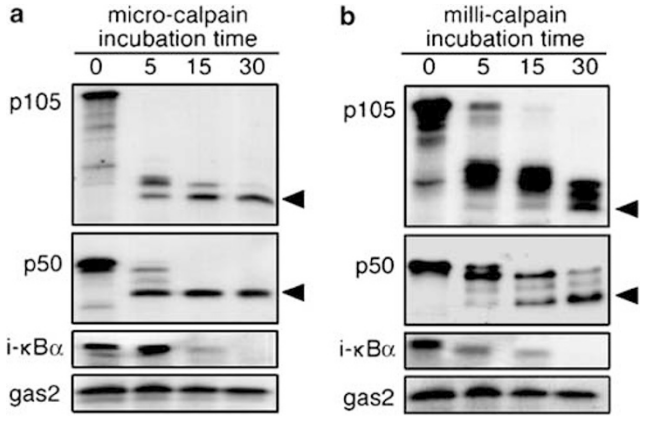

Figure 4 p105 and p50 are substrates for micro- and milli-calpain digestion *in vitro*. *In vitro* transcribed–translated ^{35}S-methionine-labelled p105, p50, I-κBα and gas2 were incubated for the indicated time intervals with commercial micro-calpain (panel **a**) or milli-calpain (panel **b**). The reactions were then stopped in Laemmli buffer and analyzed by SDS-PAGE and autoradiography. Arrowheads mark the undigested product resistant to calpain degradation

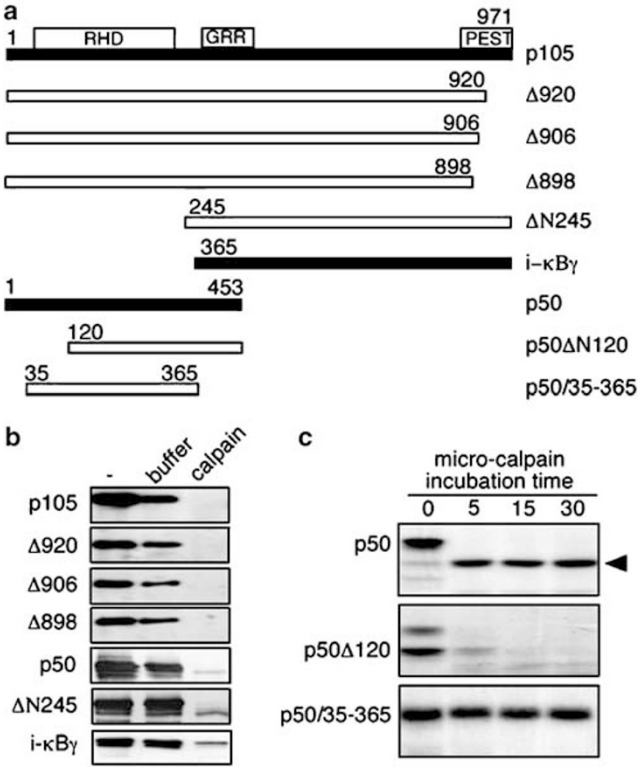

Figure 5 p50/35-365 deletion mutant is resistant to micro-calpain digestion. Panel **a**: Schematic drawing of p105, p50 and mutants. P105, Δ920, Δ906, Δ898 constructs have a GFP-tag at the C-terminal end; p50, ΔN245 and I-κBγ constructs have a HA-Tag at their N-terminal end. Domains relevant for this study are indicated on the top, namely: RHD, GRR, PEST domain. Panel **b**: Plasmids encoding for p105, Δ920, Δ906, Δ898, p50, ΔN245, I-κBγ were transfected into Phoenix cells and the overexpressed proteins were immunoprecipitated 24 h later. Each immunoprecipitation product was incubated with buffer alone or buffer plus micro-calpain. After 20 min, the reactions were stopped and analyzed by immunoblotting with antibodies specific for either GFP- or the HA-tag. In all, 20% of the input of each immunoprecipitation reaction was loaded as control. Panel **c**: p50, p50Δ120 and p50/35-365 were produced as ^{35}S-methionine-labelled proteins by *in vitro* transcription/translation and incubated for the indicated time intervals with commercial micro-calpain. The reactions were then stopped in Laemmli buffer and analyzed by SDS-PAGE and autoradiography. The arrowhead marks the undigested product resistant to calpain degradation

To gain insight into the identity of the calpain-resistant degradation product (marked by arrowheads in Figure 4), we made use of two commercial antibodies raised against specific regions of p50 and found that the N-terminal region of p50 is not retained in the calpain-resistant digestion product (data not shown). We next produced a construct encoding a polypeptide lacking the first 120 amino acids of p50, and another corresponding to Rel homology domain (RHD) which lacks sequences corresponding to the N-terminal 35 and C-terminal 88 amino acids of p50. These constructs were *in vitro* transcribed–translated and used as substrates for micro-calpain assays. As shown in Figure 5 panel c, the p50/35–365 polypeptide is resistant to calpain digestion, whereas the p50ΔN120 protein is efficiently degraded. These observations suggest that sequences between amino acids 365 and 453 corresponding to the glycine-rich region (GRR) are targeted by calpain.

p105 and p50 protein levels are modulated by calpain *in vivo*

In order to assess whether ubiquitous calpains directly regulate NF-κB1 in human cells, we analyzed the levels of the endogenous proteins after inhibition of calpain activity by two alternative approaches: siRNA-based silencing of the calpain small subunit (Capn4), which is required for the function of ubiquitous calpains, or transient overexpression of the specific natural inhibitor, calpastatin. SiRNA directed against the human *Capn4* gene reduced steady-state protein levels of the 28 kDa calpain subunit in H1299, HeLa and U2OS cells (Figure 6a). Furthermore, this Capn4-specific siRNA efficiently stabilized endogenous spectrin, a well-established calpain substrate (Figure 6a). Interestingly, Capn4 silencing was also coupled to stabilization of p105 and p50 protein levels, which strongly argues for a role of the calpain system in regulating NF-κB1 protein stability in living cells.

Overexpression of calpastatin was previously reported to stabilize other described calpain substrates.[27] Therefore, we transiently transfected H1299 and HeLa cells with a control or a calpastatin expression vector at two increasing concentrations, and analyzed endogenous p105 and p50 protein levels by Western blotting. As shown in Figure 6b, calpastatin overexpression is coupled to an increase in p105 and p50 protein levels.

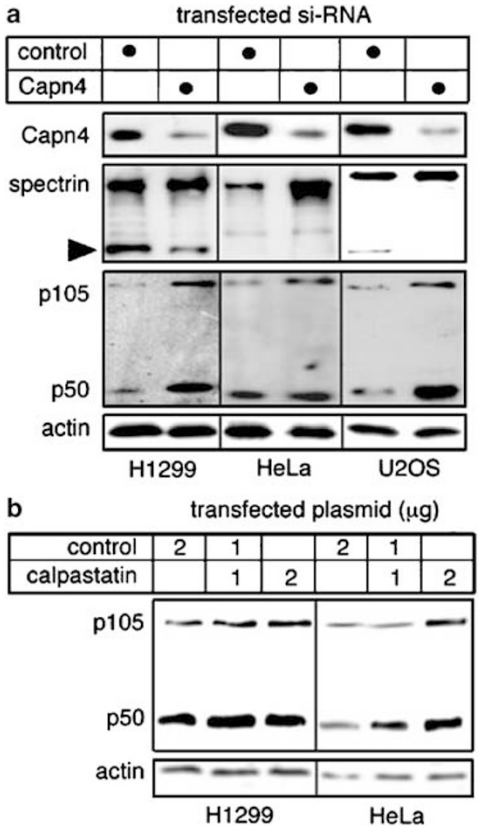

Figure 6 Capn4 silencing and overexpression of calpastatin correlate with increased p50 and p105 protein levels *in vivo*. Panel **a**: Capn4 siRNA or control siRNA were transfected in the indicated cell lines. After 48 h, lysates were prepared and analyzed by Western blotting to detect endogenous Capn4, spectrin, p105, p50 and actin as indicated. The arrowhead marks calpain cleavage product of spectrin. Panel **b**: The indicated amounts of calpastatin expression vector or empty vector were transfected into H1299 and HeLa cells. After 24 h, lysates were prepared and analyzed by Western blotting with NF-κB1-specific antibody to detect p105 and p50. Actin was analyzed as loading control

Capn4−/− MEFs are defective in NF-κB regulation by C2 ceramide

Both micro- and milli-calpain activities are undetectable in MEFs derived from Capn4−/− mice; therefore, this cellular system represents a powerful tool to help define calpain-dependent signalling and its involvement in specific cellular processes.[26] To further assess the role played by calpain in NF-κB induction by C2 ceramide, we transiently transfected wild-type and Capn4−/− MEFs with a LUC expression plasmid driven by the human iNOS promoter, a target of NF-κB. After 24 h, C2 ceramide was added and LUC activity was measured 4 and 8 h later. As shown in Figure 7a, C2 ceramide triggers the induction of the promoter in wild-type, but not in Capn4−/−, MEFs. To verify whether the differential behavior was coupled to differential NF-κB activation, wild-type control and Capn4−/−, MEFs were incubated with 100 μM C2 ceramide for 30 min or 20 h. Nuclear extracts were then prepared and incubated with a ^{32}P-labelled NF-κB consensus oligonucleotide to monitor NF-κB-binding activity. Figure 7b shows that C2 ceramide treatment correlates with an increase in NF-κB binding activity (indicated by an arrow) in wild-type cells, but not in Capn4−/− cells, in accordance with the transient transfection data shown in Figure 7a.

To investigate the molecular pathways linking ceramide signalling, calpain activation and NF-κB induction, we performed a time- course experiment of C2 ceramide stimulation in wild-type and Capn4−/− MEFs. Lysates were prepared at the indicated time points after ceramide addition and used to analyze the effect of 100 μM C2 ceramide on specific markers of calpain and NF-κB activation (Figure 7c).

C2 ceramide was previously reported to activate Raf-1/erk signalling,[3] and calpain activation by EGFR was previously shown to occur via erk signalling.[28] Therefore, we utilized an

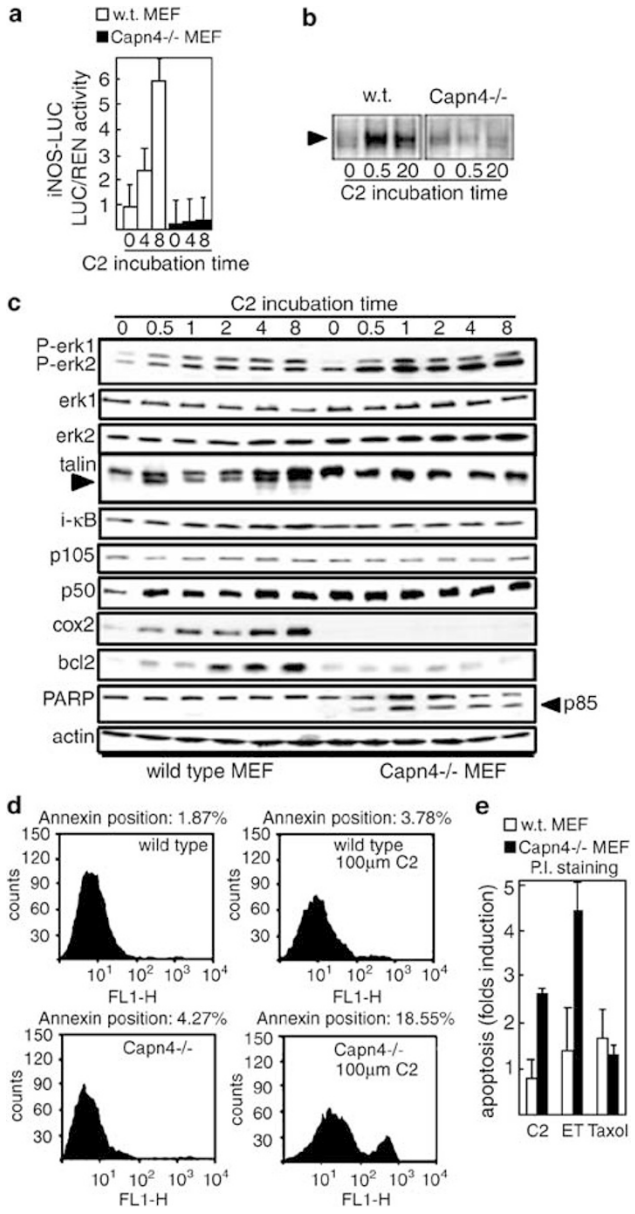

Figure 7 C2 ceramide triggers an increase in NF-κB-regulated gene expression via activation of calpain. Panel **a** wild-type and Capn4−/− MEFs were transfected with a LUC expression plasmid regulated by the human iNOS promoter and a Renilla (REN) expression vector for transfection efficiency standardization. At 24 h after transfection, C2 ceramide was added to the cells. Four and eight hours later lysates were prepared and LUC/REN activity measured. Panel **b**: Wild-type and Capn4−/− MEFs were induced for 30 min and 20 h with ceramide or left untreated and the nuclear extracts utilized to analyze NF-κB activation by means of gel shift. Arrowhead marks NF-κB binding activity. Panel **c**: Wild-type and Capn4−/− MEFs were incubated with C2 ceramide. Lysates were prepared at the indicated time points and analyzed by Western blotting with the antibodies indicated in the figure. Arrowheads mark the calpain cleavage products of talin and the caspase cleavage product of PARP. Panel **d**: Wild-type and Capn4−/− MEFs were incubated with 100 μM C2 ceramide or solvent alone for 20 h, afterwards cell death was measured by annexin V-FITC staining, followed by flow-cytometric analysis. The diagrams show the results of a representative experiment, total percentage of annexin V-positive cells is reported at the top of each diagram. Panel **e**: Wild-type and Capn4−/− MEFs were incubated with 100 μM C2 ceramide, 50 μM etoposide, 2.5 μM taxol or solvent alone for 20 h, afterwards cell death was measured by propidium iodide staining, followed by flow-cytometric analysis. The graph shows the increase in folds of the sub-G1 population after treatment with drugs with respect to control cells incubated with solvent

antibody specific for phospho-erk1/2 to monitor the signalling activated by C2 ceramide in our experimental system. A net increase in phosphorylation of erk1 and erk2 occurs both in control and in Capn4−/− fibroblasts, confirming that C2 ceramide is also activating erk signalling in our experimental settings. Interestingly, in Capn4−/− MEFs, erk1 phosphorylation is induced by C2 ceramide and then decreases, showing biphasic behavior. A possible explanation for this behavior is the stabilization of a phosphatase that is normally regulated by means of calpain-mediated cleavage.

Talin cleavage was utilized as a readout for calpain activity. We observed the appearance of the calpain-specific 190 kDa fragment 30 min after ceramide addition in wild-type fibroblasts, while in Capn4−/− cells the pattern does not change after C2 ceramide treatment, as expected.

A previous report showed that C2 ceramide triggers NF-κB activation in HL60 cells by p105 processing without affecting i-κB levels.[31] A similar mechanism may be occurring in MEFs because we did not observe C2 ceramide-induced degradation of i-κB. In wild-type MEFs, the p50/p105 ratio increases 30 min after C2 ceramide addition, followed by an increase of p105 levels. This increase is expected since the expression of p105 itself is regulated by NF-κB. Similar kinetics of protein accumulation was observed for the products of other NF-κB-activated genes, namely *COX-2* and *bcl-2*, while i-κB remains constant as previously described for HL60 cells.[31] We cannot, however, exclude a rapid degradation and re-synthesis of i-κB at a specific time-point not considered in the present study. The protein levels of p105, p50, bcl-2 and COX-2 are not significantly affected by C2 ceramide treatment in Capn4−/− cells, strongly arguing for the requirement of calpain in C2 ceramide-driven activation of NF-κB. Altogether, the data reported above indicate that the calpain-deficient Capn4−/− MEFs are defective in NF-κB activation in response to C2 ceramide-triggered signalling.

To investigate any differential cytotoxic effect of ceramide in Capn4−/− *versus* wild-type MEFs, PARP cleavage anlaysis was utilized as a readout for apoptosis. The blot shown in Figure 7c shows that PARP cleavage and apoptosis induction occur preferentially in Capn4−/− MEFs, where the NF-κB-mediated survival pathway is missing. Apoptosis induction was further studied by annexin staining, followed by FACS analysis. By this technique, we monitored apoptosis induction in Capn4−/− and control wild-type fibroblasts after 20 h incubation with C2 ceramide. The results of a typical experiment are shown in Figure 7d and demonstrate that apoptosis is occurring preferentially in Capn4−/− MEFs in agreement with the PARP cleavage data shown in Figure 7c. These results obtained in Capn4−/− MEFs are in accordance to the ones obtained by transient silencing of Capn4 by siRNA in HeLa cells (Figure 2c) and confirm that knocking down calpain activity can enhance the induction of apoptotic cell death by ceramide. In order to verify whether the lack of calpain activity determines a general increase in susceptibility to drugs or had any specificity for ceramide, we challenged Capn4−/− and control wild-type MEFs with etoposide, a known inducer of endogenous ceramide and taxol that does not induce ceramide; 100 μM ceramide and solvent alone were utilized as positive and negative controls, respectively.

After 20 h treatment, the cells were stained with propidium iodide and analyzed by FACS to detect the sub-G1 population corresponding to apoptotic cells. The graph shown in Figure 7e is representative of three independent experiments and indicate the increase in apoptosis with respect to control cells. Interestingly, the susceptibility to etoposide is higher in Capn4−/− with respect to control wild-type MEFs, as it is the case for C2 ceramide, while susceptibility to taxol is similar in the two cell lines. It must be noted that ceramide was reported to induce also alternative death styles including autophagy and necrosis; investigating the role of calpain in such processes is certainly worthwhile.

Discussion

The role of endogenous ceramide and its implication in the many signal transduction pathways in which it was reported to be a second messenger is not completely defined.[1] A very recent report demonstrated that endogenous nSMase2 levels are induced upon confluence and are involved in induction of cell cycle arrest in MCF7 cells.[40,41] Furthermore, nSMase overexpression and treatment with cell-permeable ceramide analogues can induce growth arrest in a number of cells types including SK-Hep1,[6] U937[7] and HT-29.[8]

Over 1000 research papers have described the induction of programmed cell death (apoptosis) by interventions that elevate the cell content of ceramide.[30] Pharmacological manipulation of sphingolipid metabolism to enhance tumor cell ceramide is being exploited and offers a novel approach to cancer chemotherapy.[42]

A clear-cut involvement of ceramide in apoptosis induction by radiation was demonstrated by a study using lymphoblasts from Niemann-Pick patients, which have an inherited deficiency of acid sphingomyelinase activity. These lymphoblasts fail to respond to ionizing radiation with ceramide generation and apoptosis.[43] In addition, asmase−/− MEFs failed to generate ceramide and were totally resistant to radiation-induced apoptosis and partially resistant to actinomycin and serum withdrawal. Most importantly, the sensitivity to stress could be restored in the asmase−/− MEFs by administration of natural ceramide.[44]

We observed that C2 ceramide, a cell-permeable analogue of ceramide, activates cell death in a number of human cell lines, as previously described in publications from many different laboratories. Quite paradoxically, we have found that C2 ceramide activates, at the same time, an NF-κB-dependent survival pathway through calpain, in accordance with a previous report.[24] Overall, these data argue for the existence of two parallel pathways leading to opposite cell fates. In particular, the increase in calpain activity induced by C2 ceramide leads to a rapid decrease in p105/p50 ratio, as previously described in another cellular context,[31] thus leading to activation of NF-κB and subsequent switching of κB-modulated survival genes. This survival pathway functions as a brake for the C2 ceramide-induced death pathways. We have found that calpain can process both p105 and p50 *in vitro*, whereas, as previously shown in HL60 cells,[31] we found that ceramide triggers an effect on p105 processing, and not on p50 degradation in MEFs. A likely explanation of these apparently contrasting results is that other interacting factors and/or modifications of p50 prevent its degradation in the living cell.

It is well established that resistance to cancer therapies is mediated by resistance to apoptosis, and therefore it was suggested that new approaches to cancer therapy that inhibit nuclear translocation of NF-κB may prove to be highly effective in the treatment of certain tumors.[45,46] In light of the data presented in this paper, we might envision an alternative approach involving selective inhibition of calpain to potentiate chemotherapy efficacy.

Calpain has recently emerged as a system that regulates protein stability and cell functions in parallel to the more extensively studied ubiquitin–proteasome system. A number of human diseases including neurological disorders, limb-girdle muscular dystrophy 2A, gastric cancer and type II diabetes have been associated with improper function of members of the calpain/calpastatin system.[27] Therefore, finding the substrates regulated *in vivo* by this system and elucidating the involved molecular mechanisms represent particularly urgent tasks.

Understanding the role of calpains in apoptosis is complicated by the ability of these proteases to cleave both positive and negative regulators of apoptosis. Calpains were shown to act as negative regulators of caspase processing and apoptosis by effectively inactivating upstream caspases 7 and 9.[47] Activation of calpain was shown to protect from apoptosis by inducing parkin-mediated cleavage of alpha-synuclein in neurons.[48]

In addition, calpains were shown to activate survival through the erk pathways in neurons,[49] and NF-κB survival pathway after TNF treatment.[24] On the other hand, calpain can cleave and activate caspase 12[50] and calpain-mediated cleavage of bax to a p18 form accelerates apoptosis.[51]

The regulation of the transcription factor NF-κB relies in part on tissue-specific and ubiquitous calpains through degradation of the prototypical inhibitor i-κBα.[52,34] Interestingly, recent data indicate that the NF-κB inhibitor p105 can also be processed by ubiquitin-independent alternative pathways.[53] We have demonstrated that p105 can be proteolytically processed by ubiquitous calpains *in vitro* and that specific inhibition of calpain activity is coupled to the stabilization of p105 *in vivo*. These results clearly indicate that calpain acts in parallel to the proteasome for NF-κB1 regulation, raising interesting questions about the possible cross-talk between the two proteolytic systems.

Among the p50 and p105 derivatives utilized in the mapping studies, only a p50-derived construct lacking the glycine-rich region is clearly protected from calpain-mediated degradation. It is therefore tempting to speculate that this region is involved not only in proteasome-mediated,[54,55] but also in calpain-mediated degradation. Interestingly, previous studies demonstrated that p105 could be phosphorylated *in vitro* by casein kinase II (CK2).[55] CK2 phosphorylation was recently shown to prime i-κBα for calpain-mediated degradation.[56] In addition, CK2 is a C-terminal i-κB kinase responsible for NF-κB activation during UV response[57] and promotes aberrant activation of NF-κB, transformation and survival of breast cancer cells.[58] Further studies are required to dissect the region required for calpain regulation of p105 and the involvement of CK2 in such regulation.

Materials and Methods

Chemicals and reagents

C2 ceramide and dihydroceramide C2 were purchased from Sigma-Aldrich. Lipofectamine Reagent and Oligofectamine were bought from Invitrogen Life Technologies. Calpain I and Calpain II from porcine erythrocytes were purchased from Calbiochem–Novabiochem Corporation, La Jolla, CA, USA. Custom SMARTpool for Capn4 silencing was purchased from DHARMACON, Inc. Dallas, TX, USA. Cytotoxicity assay (Cyto Tox) was purchased from Promega and utilized as recommended by vender.

Plasmids and constructs

The eukaryotic expression vector p105-PCDNA3/FLAG was a kind gift of Dr Claus Scheidereit (Berlin) and has been described before.[35] I-κBγ and p105 ΔN245 were kind gifts of Dr G Ghosh (La Jolla, CA, USA) and were described previously.[59] The plasmid containing bcl-x/LUC was a kind gift of Dr JR Perez-Polo (Galveston, TX, USA). PGL2-iNOS was a kind gift of Dr R Shapiro, Pittsburg, PA, USA. p105 cDNA and deletion mutants were PCR amplified and subcloned into the vectors pEGFP-N1.[60] p50-GFP was obtained by subcloning NF-κB1 (p50) cDNA obtained from PRSV NF-κB1 (p50) kindly donated by Dr GJ Nabel, and described in Duckett *et al*[61] into PCDNA-HA. All constructs were checked by sequence analysis.

Cell culture

Wild-type and Capn4−/− MEFs were described previously (Dourdin, 2002). p50−/− and p65−/− mouse fibroblasts were kind gifts of Dr D Baltimore. NIH-3T3, HeLa, HCT116, MCF7, U2OS, H1299 cells and the mouse fibroblasts mentioned above were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Immunological procedures

Standard protocols for immunoblotting and immunoprecipitation were used. Monoclonal antibodies against iNOS, Cox-2, bcl-2 and bcl-x were purchased from BD Biosciences Pharmingen. Antibody against NF-κB p50 (E-10) was purchased from Santa Cruz Biotechnology, Inc., SantaCruz, CA, USA. Monoclonal antibody anti-PARP was purchased from Oncogene Research Products, CN Biosciences, Inc. Monoclonal anti-spectrin was purchased from Chemicon, International.

Transfection

Transfections of Phoenix cells were performed by the calcium phosphate method using standard procedures. U2OS, HeLa, H1299, Capn4−/− and control mouse fibroblasts at 60-80% confluency were transiently transfected or oligofected using LipofectAMINE Plus reagent (Life Technologies, Inc.) or Oligofectamine according to the manufacturer's instructions.

Statistical analysis

Results are expressed as means ± standard deviation of at least three independent experiments performed in triplicate or quadruplicate, unless indicated otherwise. Statistical analysis was performed using Student's *t*-test, with level of significance set at $P<0.05$. Values of $P<0.05$ were considered to be significant.

In vitro protease assay

^{35}S-labelled *in vitro* translated protein (obtained by standard procedures) and immunoprecipitation products were incubated with 0.005 U/μl micro- or milli-calpain at room temperature following vender instructions. Buffer composition was: 30 mM Tris, pH 7.5, 1.5 mM DTT, 750 μM $CaCl_2$ for micro-calpain and 40 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM $CaCl_2$ for milli-calpain. Reactions were terminated at the indicated time points by adding SDS-PAGE loading buffer and analyzed on a 12.5% SDS-PAGE after boiling for 2 min.

Gel retardation assays

Nuclear extracts and oligonucleotide probes were prepared by standard procedures. In all, 5 μg of nuclear extracts were incubated with [γ-^{32}P]ATP- (Amersham, UK) labelled NF-κB-specific oligonucleotide and analyzed on native 5% gels. Supershift assays was performed by preincubating nuclear extracts with specific antibodies for 15 min. NF-κB p50 and p65 antibodies were from Santa Cruz Biotechnology, Inc.

Annexin V and propidium iodide staining and FACS analysis

Cells were trypsinized, fixed, stained with propidium iodide, according to standard procedures and analyzed by FACScan (Becton Dickinson). A total of 15 000 events were collected in list mode fashion, stored and analyzed by Cell Quest software.

Translocation of phosphatidyl serin to the cell surface was monitored by using an Annexin V-FITC apoptosis detection kit (Sigma). Cells were trypsinized, washed in PBS and resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM $CaCl_2$. Cell density was adjusted to 2–5 × 10^5 cells/ml. A volume of 1 μl recombinant human Annexin V-FITC/a was added to 100 μl of cell suspension; the mixture was briefly mixed and incubated for 10 min at room temperature in the dark. Afterwards, 400 μl of binding buffer was added to the cells that were then analyzed by FACScan using Cell Quest software.

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