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NF-κB protects from the lysosomal pathway of cell death

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The programme of gene expression induced by RelA/NF-κB transcription factors is critical to the control of cell survival. Ligation of 'death receptors' such as tumor necrosis factor receptor 1 (TNF-R1) triggers apoptosis, as well as NF-κB, which counteracts this process by activating the transcription of anti-apoptotic genes. In addition to activating caspases, TNF-R1 stimulation causes the release of cathepsins, most notably cathepsin B, from the lysosome into the cytoplasm where they induce apoptosis. Here we report a mechanism by which NF-κB protects cells against TNF-α-induced apoptosis: inhibition of the lysosomal pathway of apoptosis. NF-κB can protect cells from death after TNF-R1 stimulation, by extinguishing cathepsin B activity in the cytosol. This activity of NF-κB is mediated, at least in part, by the upregulation of *Serine protease inhibitor 2A* (*Spi2A*), a potent inhibitor of cathepsin B. Indeed, Spi2A can substitute for NF-κB in suppressing the induction of cathepsin B activity in the cytosol. Thus, inhibition of cathepsin B by Spi2A is a mechanism by which NF-κB protects cells from lysosome-mediated apoptosis.

Keywords: apoptosis/cathepsin/lysosome/NF-κB/serpin

Introduction

During immune and inflammatory responses, NF-κB/Rel transcription factors control cell survival (Ghosh *et al*., 1998). Normally, NF-κB heterodimers of p50-Rel A (p65) are sequestered in the cytoplasm by binding to inhibitory κB proteins (IκB), and can be activated by signals that induce sequential phosphorylation and proteolysis of IκBs. The analysis of RelA-deficient mice first revealed a role for NF-κB in suppressing apoptosis. RelA$^{-/-}$ mice die before birth due to liver apoptosis (Beg *et al*., 1995), and RelA$^{-/-}$ murine embryonic fibroblasts (MEFs) are sensitive to apoptosis caused by TNF-α. This is in contrast to wild-type MEFs and cells, which are only sensitive to TNF-α if the induction of protective factors by NF-κB is blocked by inhibitors of protein synthesis (Beg and Baltimore, 1996). The protective proteins upregulated by NF-κB act in concert to antagonize the release of mitochondrial proteins into the cytoplasm and the caspase protease cascade that leads to apoptosis (Budihardjo *et al*., 1999).

Recent evidence has revealed that, in addition to the mitochondrion, the lysosome also participates in cell death (Ferri and Kroemer, 2001). Importantly, studies with knockout mice have revealed a role for lysosomal cathepsins, notably cathepsin B, as a potent inducer of apoptosis (Guicciardi *et al*., 2000; Ferri and Kroemer, 2001; Foghsgaard *et al*., 2001). Ligation of TNF-R1 has been shown to result in the activation of the lysosomal enzymes acid sphingomyelinase and ceramidase (Schutze *et al*., 1999), and the production of the lysosomotropic detergent sphingosine which induces lysosomal breakdown (Kagedal *et al*., 2001; Werneburg *et al*., 2002). Cathepsin B is released into the cytoplasm where it activates caspase-dependent and caspase-independent pathways of cell death (Guicciardi *et al*., 2000; Ferri and Kroemer, 2001; Foghsgaard *et al*., 2001). Although the means by which cathepsins induce apoptosis are not fully elucidated, cleavage of the pro-apoptotic member of the Bcl-2 family (Bid) is a possible mechanism by which cathepsin B activates the mitochondrial pathway of apoptosis (Budihardjo *et al*., 1999; Stoka *et al*., 2001). The ability of NF-κB to block apoptosis completely implies that the transcription factor activates genes that are able to block the lysosomal pathway. However, these genes have not been identified.

Members of the superfamily of serine protease inhibitors (serpins) can modulate apoptosis through the inhibition of cytosolic executioner proteases. For example, the ovalbumin (ova) like serpins cytokine response modifier A (Crm A), from cowpox, and proteinase inhibitor 9 (PI9), from humans, are potent inhibitors of the serine protease granzyme B and so protect cells from lysis by cytotoxic lymphocytes (Quan *et al*., 1995; Sun *et al*., 1996). In addition, both proteins can also inhibit cysteine proteases such as caspases and so can protect cells from apoptosis triggered by the death receptors Fas and TNF-R1 (Komiyama *et al*., 1994; Tewari and Dixit, 1995; Zhou *et al*., 1997; Annand *et al*., 1999). Thus, given the ability of NF-κB to block TNF-α-mediated apoptosis completely, we investigated whether the transcription factor activated anti-apoptotic serpins.

The transcription of the mouse gene encoding the anti-chymotrypsin-like serpin, *Serine protease inhibitor 2A* (*Spi2A*), is induced by inflammatory stimulation and,

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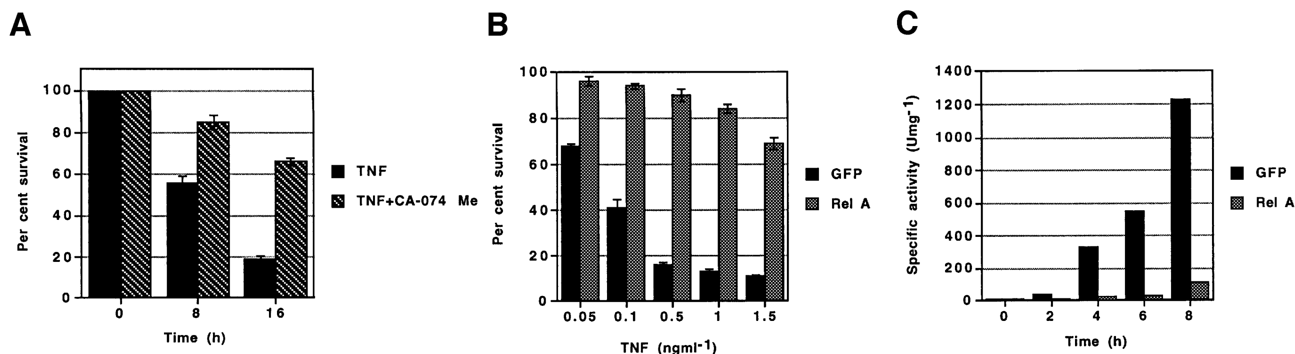

Fig. 1. NF-κB antagonizes the lysosomal pathway of cell death (**A**) Percentage survival of RelA$^{-/-}$ MEFs treatment with TNF-α (0.5 ng/ml) and CHX (0.1 μg/ml) in the presence (TNF + CA-074 Me) or absence (TNF) of CA-074 Me (30 μM). The recovery of cells was compared with those incubated with CHX alone (100% recovery) to determine the percentage of recovery. (**B**) Percentage survival of RelA$^{-/-}$ MEFs transduced by retrovirus encoding *GFP* alone or Rel A. The recovery of cells after 16 h was compared with those incubated with CHX alone (0.1 μg/ml) to determine the percentage of recovery (100% recovery). (**C**) Cathepsin B activity in crude cytoplasmic extracts from RelA$^{-/-}$ MEFs transduced by retrovirus encoding *GFP* alone or RelA after treatment with TNF-α (0.2 ng/ml) and CHX (0.1 μg/ml). This experiment is representative of two independent experiments.

uniquely for a serpin gene, depends on NF-κ-binding (Inglis *et al*., 1991; Hampson *et al*., 1997, 2001). The predicted reactive center domain of Spi2A, which is thought to interact with a target protease, is unusual for a member of the anti-chymotryspin family. Indeed, the cytosolic location of Spi2A (Morris *et al*., 2003) and the presence of cysteines at the critical P1 and P1′ positions of the reactive center are reminiscent of anti-apoptotic ova-serpins such as CrmA and PI9 (Quan *et al*., 1995; Sun *et al*., 1996). We found that complementation of RelA$^{-/-}$ MEFs with Rel A abrogates the induction of cytosolic cathepsin B after stimulation of TNF-R1, and protects against apoptosis. Purification of Spi2A revealed that it is an inhibitor of several cysteine cathepsins including cathepsin B. Importantly, we observed that Spi2A can substitute for RelA as a physiologically relevant inhibitor of cytosolic cathepsin B and a cytoprotective factor. Our findings describe a mechanism by which NF-κB suppresses the lysosomal pathway of cell death.

Results

NF-κB antagonizes the lysosomal pathway of cell death

NF-κB protects cells from TNF-α-mediated death through the upregulation of protective genes which inhibit the apoptotic cascade at several different points. A role for cathepsin B has been demonstrated in the TNF-R1-induced death of several types of tumor cells using specific inhibitors of cathepsin B, such as CA-074 Me (Foghsgaard *et al*., 2001). The complete inhibition of cathepsin B activity by CA-074 Me (30 μM) protected RelA$^{-/-}$ MEFs from TNF-α-induced death (Figure 1A). Therefore, cathepsin B activity contributes to the susceptibility of RelA$^{-/-}$ MEFs to TNF-α-induced apoptosis.

Studies in primary and tumor cells have demonstrated that activation of TNF-R1 results in the release of cathepsin B from the lysosome into the cytoplasm where it triggers apoptosis (Guicciardi *et al*., 2000; Foghsgaard *et al*., 2001; Werneburg *et al*., 2002). Using RelA$^{-/-}$ MEFs, we examined the effect of RelA complementation on the induction of cytosolic cathepsin B activity after TNF-α treatment. RelA$^{-/-}$ MEFs were transduced with retrovirus encoding RelA on a polycistronic mRNA encoding GFP (Zhang and Ren, 1998). As has been shown before, expression of RelA in RelA$^{-/-}$ MEFs (see Supplementary figure 1 available at *The EMBO Journal* Online) restored NF-κB function and gave complete protection from TNF-α-cytotoxicity (Figure 1B) (Beg and Baltimore, 1996). We next examined the influence of NF-κB/RelA on the induction of cathepsin B activity in the cytosol after treatment with TNF-α. We observed an increase in cathepsin B activity of cytosolic extracts from control RelA$^{-/-}$ MEFs as early as 2 h after treatment with TNF-α, which then increased with time (Figure 1C) . In contrast, transduction with RelA extinguished cathepsin B activity in the cytoplasm of RelA$^{-/-}$ MEFs for as long as 8 h after treatment with TNF-α (Figure 1C). Thus NF-κB may upregulate genes that inhibit cathepsin B activity in the cytosol.

Induction of Spi2A by NF-κB protects from TNF-α-mediated cell death

The transcription of *Spi2A* is induced by inflammatory stimulation and depends on NF-κ binding (Hampson *et al*., 1997, 2001; Inglis *et al*., 1991). Initially we examined whether *Spi2A* was a physiologic target of NF-κB. *Spi2A* mRNA (2.3 kb) was strongly induced by TNF-α in RelA$^{+/+}$ MEFs, but this induction was completely abolished in NF-κB/RelA$^{-/-}$ MEFs (Beg and Baltimore, 1996) (Figure 2A). While dramatic, the induction of *Spi2A* expression occurred with slower kinetics than the expression of *ikb*α, a known target of NF-κB (De Smaele *et al*., 2001). We conclude that *Spi2A* is a physiological target of NF-κB.

The control of cell survival is critically dependent on the induction of protective genes by NF-κB transcription factors (Karin and Lin, 2002). We examined whether *Spi2A* can protect RelA$^{-/-}$ MEFs from TNF-α–induced death. RelA$^{-/-}$ MEFs were transduced with retrovirus encoding *Spi2A* on a polycistronic mRNA with the *GFP* gene (Zhang and Ren, 1998). Cells from stable clones transduced with Spi2A (Spi2A cells) exhibited markedly improved survival against TNF-α, whereas cloned cells

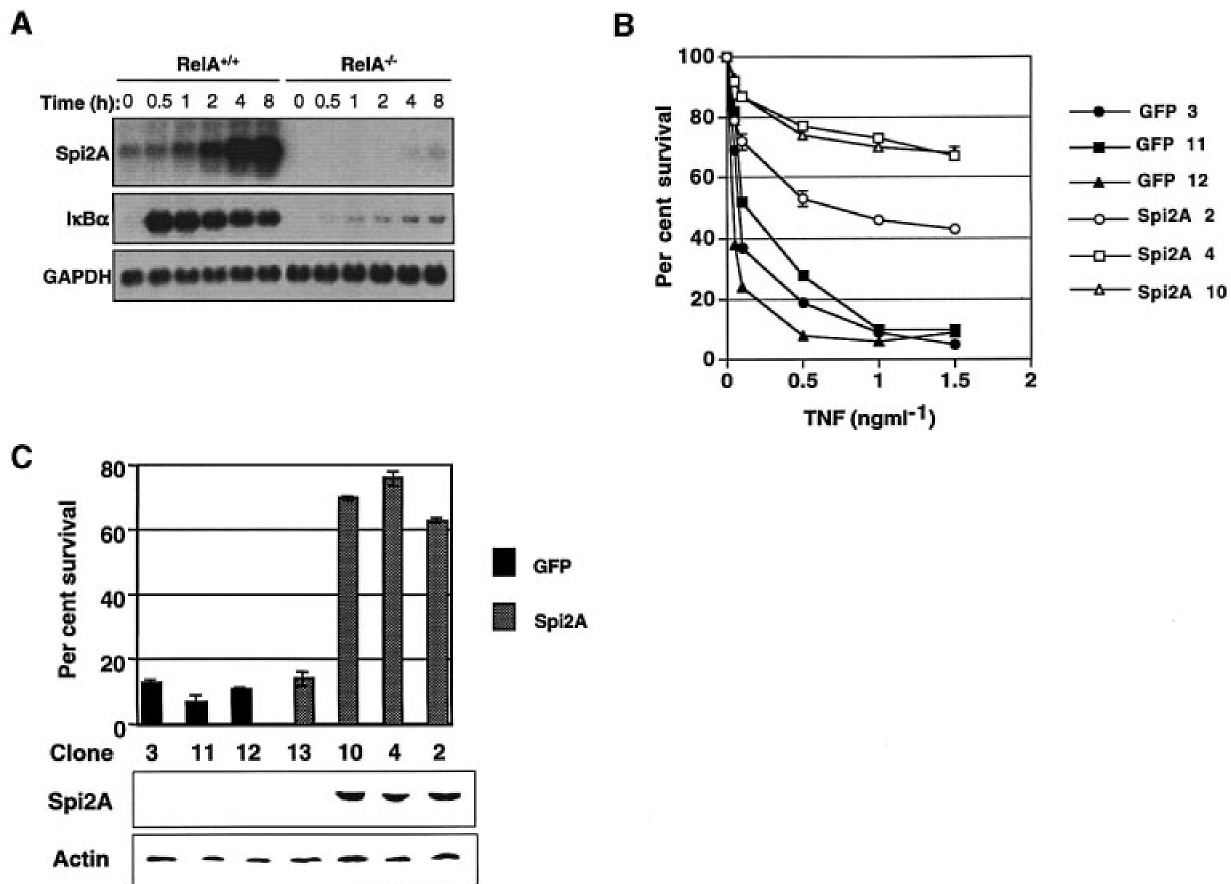

Fig. 2. Induction of *Spi2A* by NF-κB protects from TNF-α-mediated death. (**A**) Northern blots of mRNA from MEFs treated with TNF-α (0.2 ng/ml) and CHX (0.1 μg/ml) (**B**) Percentage survival of RelA$^{-/-}$ MEFs transduced by retrovirus encoding *GFP* alone or *Spi2A*. The recovery of cells after 16 h was compared with those incubated with CHX alone (100% recovery) to determine the percentage of recovery. (**C**) Western blot detection of Spi2A from GFP and Spi2A clones of RelA$^{-/-}$ MEF cells and correlation with survival after treatment with TNF-α (1 ng/ml) and CHX (0.1 μg/ml).

transduced with vector alone (GFP cells) did not (Figure 2B). Protection of RelA$^{-/-}$ MEFs from TNF-α correlated with the expression of Spi2A protein (Figure 2C). At low concentrations of TNF-α protection by Spi2A was virtually complete (Figure 2B; see 0.5 ng/ml TNF-α) and was dramatic even after 16 h at high concentrations, indicating that Spi2A can temporarily substitute for NF-κB complexes in inhibiting TNF-α-induced apoptosis.

To verify that cytoprotection mediated by Spi2A was not due to overexpression, we generated wild-type (RelA$^{+/+}$) MEFs expressing *Spi2A* in an antisense orientation (Spi2A-A cells). After treatment with TNF-α, analysis by real-time PCR revealed that the upregulation of endogenous *Spi2A* mRNA was abrogated in stable clones of Spi2A-A cells (Medhurst *et al*., 2000) (Figure 3A). Despite their ability to activate NF-κB (Supplementary figure 2), Spi2A-A cells exhibited a marked susceptibility to TNF-α-induced cell death (Figure 3B). The sensitivity of Spi2-A cells to TNF-α was also observed in the absence of cyclohexamide (CHX), indicating that TNF-α cytotoxicity was not due to an inhibition of protein synthesis in Rel A$^{+/+}$ MEFs (Supplementary figure 3). Thus Spi2A is required to antagonize TNF-α-induced apoptosis, and protection from death is a physiological function of Spi2A.

Spi2A protects from apoptosis

NF-κB protects cells from death induced by TNF-α by upregulating the expression of genes which antagonize the mitochondrial pathway of apoptosis (Beg and Baltimore, 1996; Baldwin, 2001). Given the ability of Spi2A to substitute for NF-κB complexes in protecting from TNF-α, we determined whether Spi2A could inhibit the mitochondrial pathway of apoptosis. In RelA$^{-/-}$ MEFs, TNF-α activation of caspases 3, 8 and 9, and the pro-apoptotic Bcl-2 family member Bid, was assessed by western blots (Figure 4A) and *in vitro* enzyme assays (Budihardjo *et al*., 1999; Stegh *et al*., 2000) (Figure 4B). Remarkably, the activation of both apical and executioner caspases, as well as Bid, was suppressed in RelA$^{-/-}$ MEFs that expressed high levels of Spi2A. In these cells mitochondrial depolarization, a key indicator of apoptosis, was virtually abrogated by Spi2A (Budihardjo *et al*., 1999) (Figure 4C). Importantly, Spi2A also suppressed the production of reactive oxygen species (ROS) which mediate TNF-α cytotoxicity (Goossens *et al*., 1995) (Figure 4D). Thus Spi2A abrogates TNF-α-induced caspase activation, mitochondrial depolarization and ROS production in NF-κB null cells, thereby recapitulating the effects of the transcription factor on apoptosis (Wang *et al*., 1998).

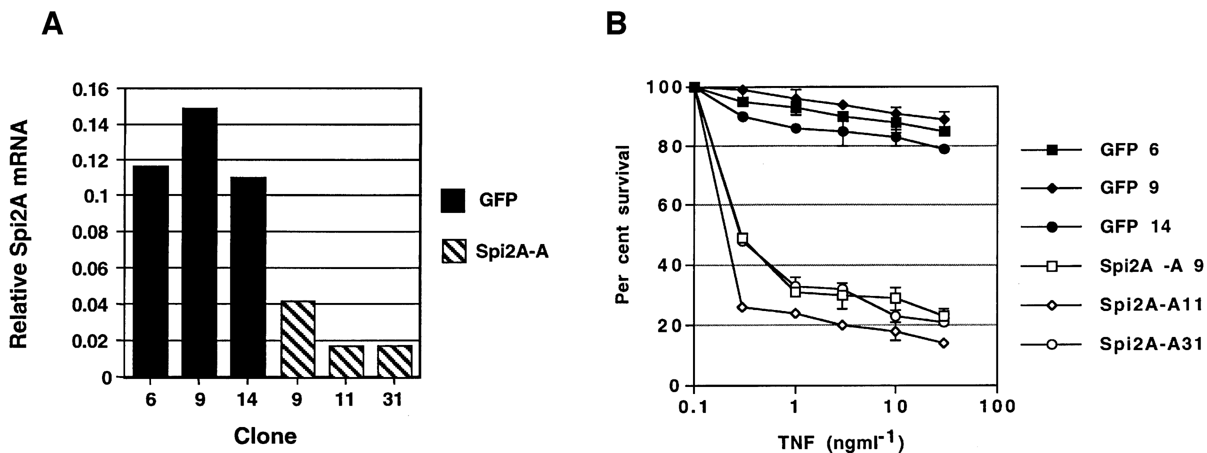

Fig. 3. Spi2A is required for the protection of wild-type MEFs from TNF-α-induced death. (**A**) Quantitation of endogenous *Spi2A* mRNA levels by real-time PCR in cloned RelA$^{+/+}$ MEFs transduced by retrovirus encoding *GFP* alone or antisense *Spi2A* (Spi2A-A) 4 h after treatment with TNF-α (10 ng/ml) and CHX(10 µg/ml). (**B**) Percentage survival of GFP clones and Spi2A-A clones of RelA$^{+/+}$ MEFs 16 h after treatment with TNF-α and CHX (10 µg/ml).

Spi2A inhibits lysosomal cysteine cathepsins

To determine the mechanism by which Spi2A antagonized apoptosis we examined the protease specificity of Spi2A *in vitro.* Spi2A was purified from RelA$^{-/-}$ MEFs transduced with retrovirus encoding epitope-tagged Spi2A (Cooley *et al.*, 2001) (Figure 5A). Spi2A inhibited both serine and cysteine proteases, similar to the serpin SQN-5 (Al-Khunaizi *et al.*, 2002). Spi2A inhibited the chymotrypsin-like serine protease cathepsin G, but not elastase or either granzyme B or granzyme A (Figure 5B). The specificity of Spi2A for cysteine proteases extended to all of the lysosomal papain-like proteases that were examined: cathepsins B, V, L, K and H. Spi2A inhibited cathepsin B with a rate constant $k > 10^6$ M^{-1} s^{-1} (Supplementary data, part 4), and so is likely to be a physiologically relevant inhibitor *in vivo* (Silverman *et al.*, 2001). However, the inhibitory effects of Spi2A did not extend to any of the caspases tested (caspases 3, 8 and 9) (Figure 5B). Thus, Spi2A is a cross-class specific inhibitor of both serine proteases and lysosomal cysteine cathepsins.

Spi2A localizes to the cytoplasm and nucleus

Spi2A is an unusual member of the chymotrypsin-like family of serpins in that it lacks a secretory signal sequence and so is likely to be located intracellularly (Hampson *et al.*, 1997). To examine further the effect of Spi2A in protecting from TNF-α-induced apoptosis we first determined the intracellular location of FLAG-tagged Spi2A in stably transduced Rel A$^{-/-}$ MEFs (Figure 2B). Immunofluorescence studies revealed staining with anti-FLAG antibodies in the cytoplasm and nucleus (Figure 6). Z-section analysis confirmed uniform distribution of anti-FLAG staining throughout the cytoplasm rather than in the plasma membrane. We conclude that Spi2A resides in the cytoplasm and nucleus. The nucleocytoplasmic localization of Spi2A revealed by our studies is concordant with findings of others with macrophage cell lines and COS cells using Spi2A antisera in immunofluorescence studies (Morris *et al.*, 2003). Localization in the cytoplasm raises the possibility that Spi2A may protect from apoptosis through the inhibition of cathepsin activity after release from the lysosome (Figure 1C).

Spi2A antagonizes the lysosomal pathway of cell death

The upregulation of *Spi2A* by NF-κB protects cells from apoptosis following ligation of TNF-R1 (Figure 2). Spi2A can inhibit cathepsin B *in vitro* (Figure 5B), and is located in the cytosol (Figure 6). Therefore the induction of Spi2A and inhibition of cathepsin B after it is released into the cytoplasm may be a mechanism by which NF-κB antagonizes the lysosomal pathway of cell death (Figure 1).

As was observed with Rel A complementation (Figure 1B), Spi2A inhibited the induction of cytosolic cathepsin B activity after treatment of Rel A$^{-/-}$ MEFs with TNF-α (Figure 7A). Direct treatment of cells with sphingosine causes the release of cathepsin B from the lysosome and the induction of apoptosis (Foghsgaard *et al.*, 2001; Kagedal *et al.*, 2001; Werneburg *et al.*, 2002). Consistent with a role in protecting from lysosome-mediated apoptosis, Spi2A could protect Rel A$^{-/-}$ MEFs from death after treatment with sphingosine (Figure 7B). Overall, we conclude that Spi2A abrogates TNF-α-induced activation of cytoplasmic cathepsin B in NF-κB null cells, thereby recapitulating the effects of the transcription factor on the lysosomal pathway of apoptosis.

Importantly, the inhibition of endogenous *Spi2A* mRNA expression by antisense *Spi2A* resulted in the induction of cytoplasmic cathepsin B activity after treatment of RelA$^{+/+}$ MEFs with TNF-α (Figure 7C). We conclude that the inhibition of cathepsin B activity in the cytosol by Spi2A is a physiologically relevant mechanism by which NF-κB protects cells from the lysosomal pathway of apoptosis.

Discussion

The program of gene expression induced by RelA/NF-κB transcription factors is critical in controlling cell survival in response to a variety of apoptotic stimuli (Baldwin, 2001). NF-κB activates multiple target genes whose

Fig. 4. *Spi2A* inhibits apoptosis induced by TNF-α. (**A**) Western blots showing the proteolytic activation of effector molecules from $RelA^{-/-}$ MEFs [GFP (clone 11) or Spi2A (clone 4)] after treatment with TNF-α (0.2 ng/ml) and CHX (0.1 μg/ml). Filled arrows indicate inactive pro-form and open arrows indicate active form of each protein. $RelA^{-/-}$ MEFs [GFP (clone 11) or Spi2A (clone 4)] were treated with TNF-α and CHX as above and the following were measured: (**B**) caspase activity, (**C**) mitochondrial depolarization and (**D**) ROS.

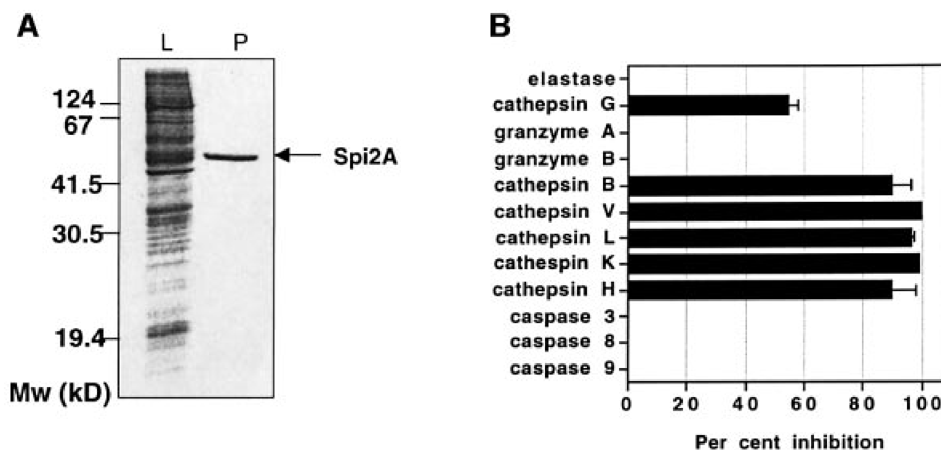

Fig. 5. The protease specificity of Spi2A. (**A**) SDS–PAGE showing Spi2A (lane P, 53 kDa) purified from lysates (lane L) of $RelA^{-/-}$ MEFs transduced with retrovirus encoding *Spi2A-3xFLAG*. (**B**) Inhibition of proteases by Spi2A. The activity of protease after preincubation with Spi2A was compared with activity from protease incubated alone (0% inhibition) and is expressed as mean ± SEM of three or four independent experiments with assays performed in duplicate.

products can block the apoptotic program triggered by death receptors or the mitochondrial pathway. NF-κB-inducible anti-apoptotic factors include those that inhibit caspase function, such as the cellular inhibitor of apoptosis proteins (c-IAPs), X-chromosome-linked IAP (XIAP) and caspase-8-c-FLIP (FLICE inhibitory protein), those that

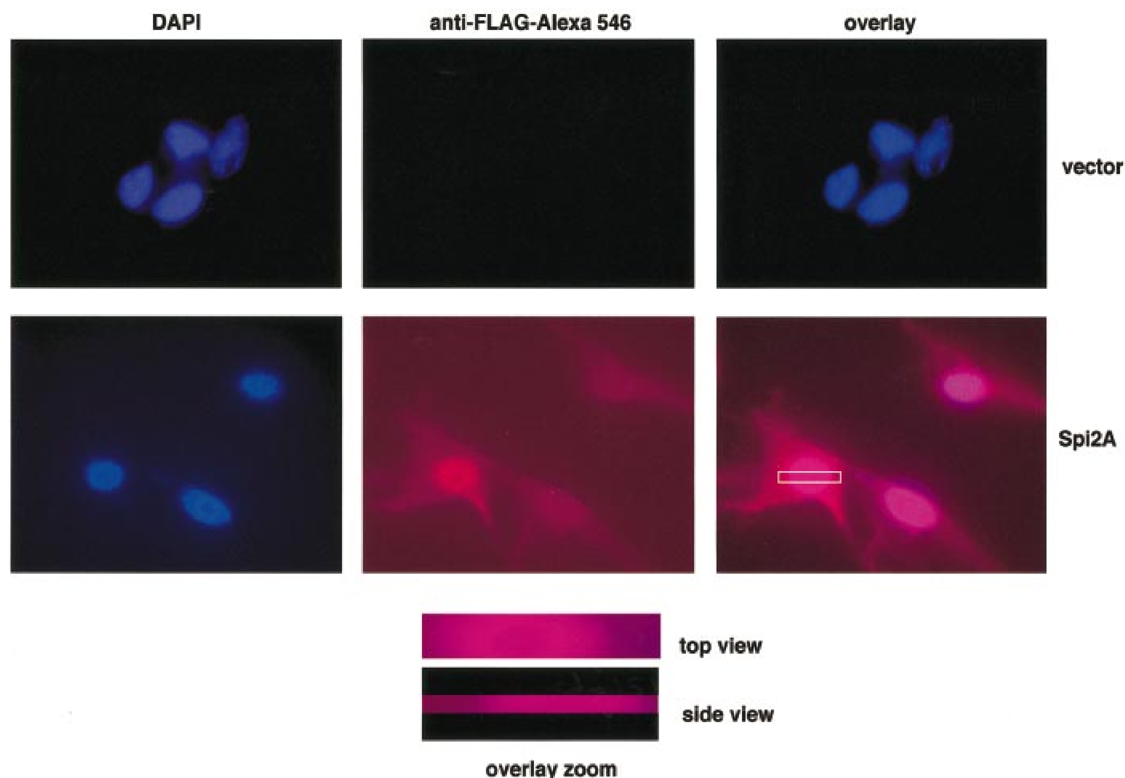

Fig. 6. Intracellular localization of Spi2A. Vector (GFP clone 11, top) or Spi2A transfected (Spi2A clone 4, bottom) Rel $A^{-/-}$ MEFs were stained for FLAG (middle panel) or DAPI (left panel); the right panel shows the overlay of the two channels. Overlay zoom, top and side views. In both views, the FLAG label appears to be uniformly distributed through the cytoplasm.

inhibit NF-κB signaling after TNF-R1 stimulation, such as TNF-R-associated factors 1 and 2 (TRAF1 and TRAF2), those that preserve mitochondrial function, such as Bcl-x_L and A1/Bf1, and those that inhibit pro-apoptotic signaling by the c-Jun kinase (JNK) pathway, such as Gadd45β and XIAP (Karin and Lin, 2002). Stimulation of TNF-R1 induces the breakdown of the lysosome and the induction of apoptosis by cathepsins released into the cytoplasm (Guicciardi *et al*., 2000; Ferri and Kroemer, 2001; Foghsgaard *et al*., 2001). We report a novel mechanism by which NF-κB protects cells against TNF-α-induced apoptosis: inhibition of the lysosomal pathway of apoptosis. The upregulation of *Spi2A* and inhibition of cytoplasmic cathepsin B, after release from lysosomes, represents a physiologically relevant mechanism by which NF-κB blocks the lysosomal pathway of cell death in MEFs. The wider physiological importance of cytoprotection from TNF-α by Spi2A is suggested by the finding that the *Spi2A* gene is located in the 'TNF protection locus' on chromosome 12 in mice (Libert *et al*., 1999).

Several observations lead us to conclude that the induction of Spi2A by NF-κB protects cells from TNF-α by inhibiting the lysosomal pathway of apoptosis. Inhibition of cathepsin B by CA-074 Me protects $RelA^{-/-}$ from TNF-α-induced apoptosis, confirming the observations that cathepsin B plays a direct role in apoptosis in other cell types (Guicciardi *et al*., 2000; Foghsgaard *et al*., 2001). Treatment of $RelA^{-/-}$ MEFs with TNF-α leads to the induction of cytosolic cathepsin B activity after only 2 h (Figures 1C and 7A). This precedes the onset of apoptosis, as measured by caspase or Bid activation (Figure 4A and B), mitochondrial depolarization (Figure 4C) and ROS production (Figure 4D), by 2 h. Therefore, in our system, the release of cathepsin B into the cytoplasm does not seem to be a consequence of apoptosis but rather precedes it. We demonstrate that Spi2A is a broad specific inhibitor of lysosomal cathepsins, including cathepsin B, and is located in the cytoplasm (Figures 5B and 6). Therefore the suppression of cathepsin B activity by Spi2A is at least one mechanism by which NF-κB antagonizes the lysosomal pathway of apoptosis.

We observed that Spi2A can partially inhibit the increase in the pH of lysosomes that occurs after TNF-α treatment, as assessed by the appearance of acridine orange (AO) low cells (Kagedal *et al*., 2001) (Supplementary figure 4). This could mean that, in addition to inhibiting cathepsin B activity in the cytoplasm, Spi2A may also prevent lysosomal breakdown. In the light of a recent study that suggests a direct role for cathepsin B in lysosomal breakdown (Werneburg *et al*., 2002), there is a possibility that Spi2A acts to preserve lysosomal integrity. Spi2A is located in the cytosol, but it is not clear whether lysosomal or cytoplasmic cathepsin B catalyzes lysosomal breakdown. Direct measurement of cathepsin traffic out of the lysosome in Spi2A-expressing Rel $A^{-/-}$MEFs will be required to test this possibility when suitable reagents become available.

The relationship between the lysosomal and caspase pathways of apoptosis is not fully elucidated and seems to

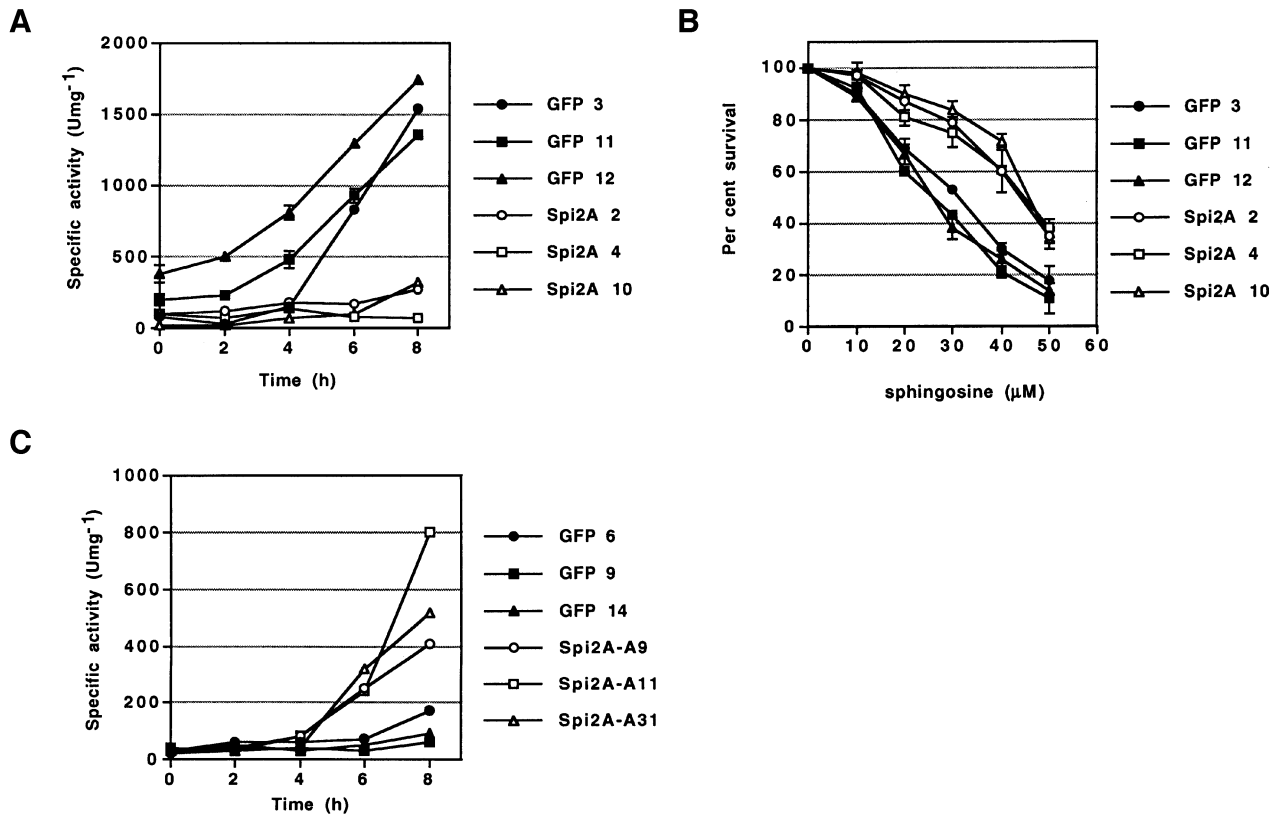

Fig. 7. Spi2A antagonizes the lysosomal pathway of cell death. (**A**) Cathepsin B activity in crude cytoplasmic extracts from cloned RelA$^{-/-}$ MEFs transduced by retrovirus encoding *GFP* alone or *Spi2A* after treatment with TNF-α and CHX as described before. (**B**) Percentage survival of *GFP* and *Spi2A* clones of RelA$^{-/-}$ MEFs 2 h after treatment with sphingosine. (**C**) Cathepsin B activity in crude cytoplasmic extracts from cloned RelA$^{+/+}$ MEFs transduced by retrovirus encoding *GFP* alone or antisense *Spi2A* (Spi2A-A) after treatment with TNF-α (10 ng/ml) and CHX (10 μg/ml).

depend on the nature of the apoptotic signal and cell type in question (Ferri and Kroemer, 2001; Foghsgaard *et al.*, 2001). After cleavage by caspase 8, Bid activates the mitochondrial pathway of apoptosis (Budihardjo *et al.*, 1999). Importantly, cathepsin B can cleave Bid but not pro-caspases *in vitro*, and so Bid may also be a possible link between the lysosomal and mitochondrial pathways of apoptosis (Stoka *et al.*, 2001). Spi2A cannot directly inhibit caspases 3, 8 or 9, and yet caspase activity in RelA$^{-/-}$ MEFs treated with TNF-α is abrogated by *Spi2A* expression (Figures 4A and B, and 5B). After ligation of TNF-R1, Spi2A inhibits cytoplasmic cathepsin B activity and so may prevent apoptosis by inhibiting the cleavage of Bid by cathepsin B and subsequent caspase activation by proteins released from mitochondria (Scaffidi *et al.*, 1998). The interconnectedness of the lysosomal and mitochondrial pathways of apoptosis and the ability of damaged mitochondria to feedback and activate caspase 8 (Scaffidi *et al.*, 1998) likely account for the ability of Spi2A to restore so robustly the NF-κB-phenotype of resistance to TNF-α to Rel A$^{-/-}$ MEFs. However, compared with other protective genes induced by NF-κB, Spi2A is certainly not unique in this regard (De Smaele *et al.*, 2001).

The inhibition of papain-like cysteine cathepsins has been observed not only for Spi2A (Figure 5B) but also for other serpins, such as squamous cell carcinoma antigen 1 (SCCA1) from humans (Schick *et al.*, 1998) and SQN-5 from mice (Al-Khunaizi *et al.*, 2002). Unlike other inhibitors of executioner protease, such as c-IAPs which inhibit caspases (Deveraux *et al.*, 1998) and cystatins which inhibit papain-like cysteine cathepsins (Turk and Bode, 1991), serpins characteristically act as 'suicide substrates' and inactivate proteases through the formation of a 1:1 covalent complex (Silverman *et al.*, 2001). The mechanism by which Spi2A inhibits cathepsins may be by acting as a suicide substrate because Spi2A can be cleaved by cathepsin G, cathepsin B and cathepsin K *in vitro* (Morris *et al.*, 2003; S.M.Raja, C.J.Froelich and P.G.Ashton-Rickardt, unpublished data). However, whether inhibition of cysteine cathepsins by Spi2A is mediated by the formation of covalent complexes, as with some but not all cross-class specific serpins, remains to be determined (Komiyama *et al.*, 1994; Annand *et al.*, 1999; Al-Khunaizi *et al.*, 2002).

Despite the fact that the mechanism by which Spi2A inhibits pro-apoptotic cathepsins is not fully elucidated, antisense experiments show that Spi2A is a physiologically relevant inhibitor of cytoplasmic cathepsin B (Figure 7C). Furthermore, we demonstrate that cathepsin B is a mediator of TNF-α-induced apoptosis in RelA$^{-/-}$ MEFs (Figure 1A). Spi2A inhibited every cysteine cathepsin we tested, and so it is possible that the protective function of Spi2A may extend to the inhibition of other pro-apoptotic cathepsins. Indeed, the inhibition of cathepsins by other serpins seems to protect cells from TNF-α-induced apoptosis. For example, the overexpression of the

human serpin SCCA 2 can inhibit TNF-α-induced apoptosis of HeLa cells possibly through inhibition of cathepsin G (McGettrick *et al.*, 2001). Although our studies support an anti-apoptotic mechanism by which Spi2A inhibits cathepsin B activity in the cytoplasm, Spi2A in the nucleus may also protect from apoptosis. Cathepsin B has been localized to the nuclear membrane in human tumor cells and so, like cystatin B, Spi2A may protect cells by inhibiting the activity of papain-like cathepsins in the nucleus (Speiss *et al.*, 1994; Riccio *et al.*, 2001).

The loss of lysosome integrity and the release of cathepsins and other digestive enzymes is a critical event in the induction not only of apoptosis but also of coagulative necrosis (Wyllie *et al.*, 1981; Ferri and Kroemer, 2001). Therefore one could predict that induction of Spi2A and NF-κB may provide protection from necrosis as well as apoptosis. Further investigation of the potential role of NF-κB and Spi2A in affording protection from necrosis during vertebrate development (Chautan *et al.*, 1999) and the pathogenesis of disease (Wyllie *et al.*, 1981), using *in vivo* models, will shed light on this potentially exciting new aspect of NF-κB biology.

Materials and methods

Spi2A mRNA expression

Total RNA (4 μg) was extracted from MEFs after treatment with TNF-α (0.2 ng/ml) (R&D) and cyclohexamide (CHX) (0.1 μg/ml) using Trizol Reagent according to the manufacturer's instructions (Invitrogen) and northern blots were prepared using standard procedures (Sambrook *et al.*, 1989). Blots were probed with an [α-^{32}P]dCTP-hexamer-labeled cDNA probe encoding *Spi2A* (Hampson *et al.*, 1997). Blots were stripped and reprobed with similarly labeled control probes encoding *IκBα* or *GAPDH* (De Smaele *et al.*, 2001).

In *Spi2A*-antisense experiments, the level of *Spi2A* mRNA was quantitated by real-time PCR using primers and probes specific for *Spi2A* (forward primer 5′-AACCAGAGACCCTGAGGAAGTG-3′; reverse primer 5′-AACTTGGGCAGGCGCAG-3′; probe 5′-AAGAACTC-TCTGAAGCCCAGGATGATACATGA-3′) (Inglis *et al.*, 1991) and the *cyclophilin A* housekeeping control gene (Medhurst *et al.*, 2000) (forward primer 5′-CCATCAAACCATTCCTTCTGTAGC-3′; reverse primer 5′-AGCAGAGATTACAGGACATTGCG-3′; probe 5′-CAGGAGAGCGT-GCCTACCCCATCTG-3′) (Megabases Inc). Probes were labeled with the fluorescent reporter dye FAM. Four hours after treatment with CHX (10 μg/ml) and TNF-α (10 ng/ml), RNA was extracted from RelA$^{+/+}$MEFs using Trizol Reagent (Invitrogen) and then cDNA was generated using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR reactions were carried out using TaqMan Universal PCR Master Mix according to the manufacturer's recommended protocol (PE Applied Biosystems) and analyzed on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Data were captured and analyzed using Sequence Detector software (PE Applied Biosystems). The slope of the standard curve describes the efficiency of the real-time PCR, which allowed us to ensure that the real-time PCR reactions consistently ran at >90% efficiency. The relative concentration of *Spi2A* RNA was calculated by dividing the concentration of *Spi2A* RNA by that of the *cyclophilin A* control gene (Hasel and Sutcliffe, 1990).

Retroviral transduction of MEFs

The cDNA for human RelA (p65) was subcloned into the Hpa 1 restriction site of the MIGR1 retroviral vector in the forward orientation (Franzoso *et al.*, 1996; Zhang and Ren, 1998). The *Spi2A* open reading frame (ORF) was amplified by PCR from cDNA prepared from purified T cells using a forward primer (5′-AGAATTCGCCACCATGGCTG-GTGTCTCCCCTG-3′) and a reverse primer (5′-TGTGGATCCTCCC-TGTCAAATCAGGCAGCATAGCGGAT-3′). These primers introduced 5′ *Bam*HI and 3′ *Eco*R1 restriction sites and mutated the stop codon of *Spi2A* ORF to facilitate the production of an inframe fusion protein between Spi2A and C-terminal 3xFLAG (22 amino acids) after cloning into the 3xFLAG-CMV-14 expression vector (Sigma-Aldrich). Using the same forward primer and a reverse primer specific for 3xFLAG DNA that introduced an *Eco*R1 restriction site (5′-GTGAATTCATCACTAC-TTGTCATCGT-3′), the S*pi2A-3xFLAG* ORF was amplified by PCR and then subcloned into the *Eco*R1 site of the MIGR1 retroviral vector in the forward or reverse orientations. The MIGR1 retroviral vector directed the expression of RelA, *Spi2A-3xFLAG* or *Spi2A-3xFLAG* antisense mRNA as a biscistronic mRNA encoding GFP.

Retrovirus was produced as described previously (Burns *et al.*, 1993). Briefly, cells of the 293 GP packaging line (4×10^6) were transiently transfected with *MIGR1-Spi2A-3xFLAG* DNA (6 μg) and DNA-encoding vesicular stomatis virus (VSV) glycoprotein (6 μg) using Lipofectamine PLUS reagent according to manufacturer's instructions (Invitrogen). After 48 and 72 h supernatant containing retrovirus was harvested, filtered and stored at –80°C until needed. MEFs ($1–2 \times 10^5$) were seeded in six-well plates and transduced with 4 ml of retroviral supernatant containing polybrene (8 μg/ml) by centrifugation at 1000 *g* for 1 h at room temperature, followed by incubation at 37°C for 24 h. After 48 h, the transduction efficiency was determined by measuring the percentage of GFP-positive MEFs by FACS, which was routinely 96–98%. Transduced MEFs that were in the top 5% of GFP expression were purified by FACS and cloned.

Fluorescence microscopy

RelA$^{-/-}$ MEFs were transduced with retrovirus encoding S*pi2A-3xFLAG* or empty vector and plated in a Chamber-Slide (Lab-Tek, Nalge Nunc) overnight at 10 000 cells per chamber in 10% fetal calf serum (FCS) containing Dulbecco's modified Eagle's medium (DMEM). Immunofluorescence localization for Spi2A was performed using the anti-FLAG antibody (Sigma). Briefly, the cells were washed three times with chilled phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA)–PBS for 15 min at room temperature (RT), permeabilized using 0.5% Triton X-100 (15 min at RT) followed by five washes with chilled PBS. The slides were blocked with 2% normal mouse serum (NMS) in PBS (45 min at RT) followed by incubation (90 min at RT) with the biotinylated anti-FLAG antibody (10 μg/ml). After washing off the unbound antibody, the slides were incubated (60 min at RT) with Streptavidin (SA)–Alexa 546 (1 μg/ml; Molecular Probes) followed by washes with chilled PBS. Finally the cells were mounted in Vectashield mounting medium (Vector Labs) containing DAPI as the nuclear stain. The cells were observed and imaged on a Leica DMIRE2 inverted microscope equipped with a Photometrics CoolSNAP HQ digital camera (Roper Scientific). Fluorescence images of each of the fluorophores were acquired sequentially using the following Chroma filter cubes: DAPI, cube 31000 (Ex. 340–380 nm, Em. 435–485 nm); FLAG, cube 41004 (Ex. 535–585, Em. 610–680 nm). Images were acquired and then overlaid using Meta Imaging Series software version 4.6.5 (Universal Imaging Corporation). To determine whether FLAG was distributed throughout the cytoplasm (rather than being bound to the plasma membrane), Z-series of individual cells were captured and deconvolved using MetaMorph. A three-dimensional model was reconstructed from the Z-series, and then sliced and rotated (again using MetaMorph) to obtain a side view.

Protein expression

Antiserum specific to Spi2A peptides [peptide 1 (amino acids 406–423), NH_2-(C) NPERSTNFPNGEGASSQR-COOH; peptide 2 (amino acids 278–294), NH_2-(C) SLQPETLRKWKNSLKPR-COOH] was raised in rabbits using standard procedures (Coligan *et al.*, 1995). Briefly, two rabbits were immunized with each peptide conjugated to KLH and then boosted twice with immunogen over a period of 3 months. Anti-Spi2A antibodies were affinity purified on columns of immunizing peptide, eluted in 3M KSCN and then dialyzed against PBS.

Detergent extracts from RelA$^{-/-}$ MEFs transduced with control or retrovirus encoding Spi2A were resolved by SDS–PAGE and then immunoblotted (25 μg per lane) and probed with either peptide-1- or peptide-2-specific anti-Spi2A antibodies (10 μg/ml) using standard protocols (Coligan *et al.*, 1995). Spi2A was detected after probing with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich) at 2 μg/ml and chemiluminescence (ECL-kit, Amersham). Antibodies purified from the two rabbits immunized with either peptide 1 or peptide 2 detected Spi2A as a 52 kDa protein in extracts from Spi2A cells but not GFP cells. To control for equal loading, blots were stripped and reprobed for actin (42 kDa) with anti-actin monoclonal antibody clone ACTN05 (RDI Research Diagnostics Inc) at 0.5 μg/ml and anti-mouse IgG-HRP (Sigma-Aldrich) at 2 μg/ml.

Survival assays

Although RelA$^{-/-}$ MEFs are markedly more sensitive to TNF-α than RelA$^{+/+}$ MEFs, we still used low levels of CHX (0.1 μg/ml) in our survival assays. This was to suppress any protective activity of constitutively active non-RelA NF-κB molecules that are present in RelA$^{-/-}$ MEFs (Figure 4). Thus RelA$^{-/-}$ MEFs were treated with CHX and TNF-α (R&D) and the number of live GFP-positive adherent cells was counted by flow cytometry after 16 h, if not indicated otherwise. Live cells were defined as those that excluded propidium iodide (PI-negative) and had the appropriate size, as defined by forward- and side-scatter characteristics (Coligan *et al*., 1995). For RelA$^{+/+}$ MEFs, TNF-α cytotoxicity was determined after 16 h with CHX (10 μg/ml), if not indicated otherwise. Cathepsin B activity was inhibited by a 1 h pretreatment of MEFs with CA-074 Me (30 μM) (Peptide Institute). Complete inhibition of cathepsin B activity was verified by enzyme assay. RelA$^{-/-}$ MEFs were treated with 10–50 μM sphingosine (Calbiochem) and the number of live GFP-positive adherent cells was counted by flow cytometry after 2 h.

Death effector assays

Death effector pathways were induced in RelA$^{-/-}$ MEFs by treatment with TNF-α (0.2 ng/ml) and CHX (0.1 μg/ml). Assays for executioner proteases (caspases and cathepsin B) were performed on crude cytoplasmic extracts (Stegh *et al*., 2000). Briefly, MEFs (10^6) were lysed in 10 mM Tris–Cl pH 7.5, 100 mM NaCl, 1 mM EDTA and 0.01% Triton X-100 (50 μl) for 30 min on ice, and then centrifuged at 15 000 *g* for 30 min at 4°C and the supernatant recovered. Protein concentration was determined by Lowry assay (DC-protein assay kit, Bio-Rad). Western immunoblots were performed on crude cytosolic extracts (50 μg per lane) using standard protocols and probed with the following antibodies: goat anti-mouse Bid antiserum (1 μg/ml) (R&D systems), rabbit anti-human caspase 9 antiserum (2 μg/ml) (Cell Signaling Technology), rabbit anti-human caspase 3 antiserum (2 μg/ml) (Cell Signaling Technology) and mouse anti-human caspase 8 monoclonal antibody clone 12F5 (1 μg/ml) (Axxora). The following secondary antibodies were used: anti-goat IgG HRP (0.5 μg/ml) (Santa Cruz Technology), anti-rabbit IgG HRP (0.5 μg/ml) (Amersham), anti-mouse IgG HRP (0.5 μg/ml) (Santa Cruz Technology). Specific proteins were visualized using chemiluminesence (ECL-kit, Amersham).

Colorimetric assays for caspases were performed in reaction buffer [50 mM HEPES pH 7.4, 100 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM EDTA, 10% glycerol, 0.1% CHAPS] at 37°C on crude cytoplasmic extracts using *p*-nitroaniline (*p*NA)-labeled substrates (Calbiochem) specific for caspases 3 and 7 (Ac-DEVD-*p*NA), caspase 8 (Ac-IETD-*p*NA) and caspase 9 (Ac-LEHD-*p*NA), each at 0.2 mM. Specific activity was determined by subtracting the apparent activity detected after preincubation of extract for 1 h with the pan-caspase inhibitor Z-VAD.fmk (50 μM) (ICN Biomedicals Inc.) and then normalizing for the amount of protein. Assays for cathepsin B activity in crude cytoplasmic extracts was performed in reaction buffer (100 mM $KHPO_4$ pH 6.1, 2 mM DTT, 1 mM EDTA) at 37°C using the Z-RR-*p*NA substrate (Calbiochem), which is specific for cathepsin B but not for other cysteine cathepsins from the lysosome (Barrett and Kirschke, 1981), at 0.4 mM. Specific activity was determined by subtracting the apparent activity detected after 30 min preincubation of extract at 37°C with the cathepsin B inhibitor CA-074 Me (Peptide Institute) at 30 μM and then normalizing for the amount of protein.

Mitochondrial membrane potential and ROS production were measured using the fluorescent dyes JC-1 (3 μg/ml) and dihydroethidium (HE) (5 μM) (Molecular Probes), respectively, and flow cytometry according to the manufacturer's instructions.

Protease specificity of Spi2A

RelA$^{-/-}$ MEFs were transduced with retrovirus encoding *Spi2A* with a C-terminal *3 xFLAG* epitope tag and Spi2A-3xFLAG purified using the method described previously (Cooley *et al*., 1998). Briefly, cells (3×10^9) were lysed and Spi2A-3xFLAG (75 μg) was purified by batch Q-Fast Flow ion-exchange chromatography (Pharmacia Biotech) after elution at 160–220 mM NaCl followed by anti-FLAG antibody columns, performed according to the manufacturer's instructions (Sigma-Aldrich). Spi2A-3xFLAG was dialyzed into PBS and stored as aliquots at –80°C until needed.

Proteases were purchased from the manufacturers (Calbiochem or Athens Research and Technology) except for granzymes A and B which were purified as described (Hanna *et al*., 1993) and cathepsins V and K which were purified as described (Linnevers *et al*., 1997; Bromme *et al*., 1999). Proteases (20 nM) were incubated in the appropriate assay buffer with Spi2A at 200 nM (at least 10-fold excess of inhibitor to maintain pseudo-first-order conditions) for 1 h at 37°C. Control samples included only the enzyme, without the inhibitor. After 1 h protease activity was assayed. For serine proteases the following substrates (Calbiochem) were used at 1 mM (Cooley *et al*., 2001; Al-Khunaizi *et al*., 2002): human cathepsin G, Suc-AAPF-*p*NA; human elastase, MeOSuc-AAPV-*p*NA in assay buffer (20 mM Tris–HCl pH 7.4, 500 mM NaCl, 0.1% PEG); human granzyme B, IETD-*p*NA; human granzyme A, BLT-*p*NA. For cysteine cathepsins the following substrates (Molecular Probes) were used at 5 μM: human cathepsins B, L, K and V, $(Z\text{-}FR)_2$-R110; human cathepsin H, $(Z\text{-}PR)_2$-R110 in assay buffer (50 mM NaAc pH 5.4, 4 mM DTT,1 mM EDTA) (Al-Khunaizi *et al*., 2002). Substrate hydrolysis was measured in a fluorescence microtiter plate reader (Spectramax Gemini XS, Molecular Devices). Percentage inhibition was calculated from the residual enzyme activity compared with no Spi2A controls. Incubation with alkaline phosphatase tagged with C-terminal 3 x FLAG (Sigma-Aldrich) under the same conditions had no effect on protease activity.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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