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Apoptosis of hematopoietic cells induced by growth factor withdrawal is associated with caspase-9 mediated cleavage of Raf-1

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The Raf-1 serine/threonine kinase is a key protein that is implicated in the transmission of many growth and cell survival signals. In the present study we demonstrate that apoptosis of hematopoietic cells induced by IL-3-deprivation is associated with the cleavage of Raf-1, resulting in the separation of the N-terminal regulatory domain and the C-terminal kinase domain. Raf-1 cleavage specifically occurs upon triggering of the mitochondrial death pathway, and coincides with the activation of specific caspases. Moreover, Bcl-2 overexpression or treatment with the caspase inhibitor z-VAD.fmk completely prevented Raf-1 cleavage, whereas caspase inhibition by treatment of cells with Ac-DEVD.fmk or z-IETD.fmk, or CrmA overexpression had no effect. Furthermore, *in vitro* cleavage studies indicate that caspase-9, which is the apical protease in the mitochondrial death pathway, is able to cleave Raf-1 at position D279. Cell fractionation studies showed that the Raf-1 C-terminal fragment that is generated upon IL-3 withdrawal is localized predominantly to the mitochondria. In addition, constitutive expression of this C-terminal Raf-1 fragment fused to a mitochondrial targeting sequence in Ba/F3 pre-B cells significantly delays apoptosis induced by IL-3 withdrawal. These results suggest an important role for caspase-9 mediated cleavage of Raf-1 in the negative feedback regulation of hematopoietic cell apoptosis induced by growth factor withdrawal.
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Introduction

A common feature of apoptosis is the activation of caspases. These intracellular aspartic acid-directed proteases are synthesized as inactive precursors that are activated by dimerization or proteolytic processing (Boatright *et al.*, 2003; Boatright and Salvesen, 2003). They act either as initiators (caspases-2, -8, -9) in response to apoptotic signals, or as effectors (caspases-3, -6, -7) that finally cleave a number of vital proteins (Earnshaw *et al.*, 1999). Many apoptotic responses are initiated by activation of the apical caspases-8 or -9, the former by recruitment to ligated cell surface receptors belonging to the tumor necrosis factor receptor-1 family, and the latter by recruitment to Apaf-1 in the presence of dATP following delivery of cytochrome *c* from mitochondria (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Li *et al.*, 1997). In this regard, at least two major pathways for caspase activation and apoptosis have been delineated, including an 'extrinsic' pathway triggered by the TNF family death receptors and an 'intrinsic' pathway activated by damage to mitochondria leading to cytochrome *c* release into the cytosol. However, apoptotic stimuli do not often act through a single signaling pathway. In so-called 'type II' cells, the death receptor and the mitochondrial pathway are interconnected via caspase-8-mediated translocation of truncated Bid (tBid) to mitochondria, where tBid triggers cytochrome *c* release into the cytosol (Li *et al.*, 1998; Luo *et al.*, 1998; Scaffidi *et al.*, 1998).

Raf-1 is a serine/threonine kinase implicated in cell survival. Structural and functional studies have shown that Raf-1 is composed of two distinct domains, an N-terminal regulatory domain (containing the conserved regions CR1 and CR2) and a C-terminal kinase domain (constituting the third conserved region, CR3). The amino-terminal domain suppresses the catalytic activity of Raf-1, and its deletion constitutively activates Raf-1 (Stanton *et al.*, 1989; Heidecker *et al.*, 1990).

Raf-1 was originally described as the initiator of a mitogen-activated protein kinase (MAPK) cascade involved in the regulation of cell proliferation and differentiation (Davis, 1994; Robinson and Cobb, 1997). Surprisingly, three independent Raf-1 knockouts in

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mice, generated with different gene targeting strategies, showed normal MAPK activation in embryonic fibroblasts (MEFs), suggesting that Raf-1 may be dispensable for MAPK signaling (Wojnowski *et al.*, 1998; Huser *et al.*, 2001; Mikula *et al.*, 2001). However, based on the increase in apoptosis in mutant embryonic tissues and the increased sensitivity of Raf-deficient mouse embryonic fibroblasts to apoptotic agents, it was concluded that the primary function of Raf-1 is to protect cells from apoptosis independently of the MAPK cascade. Consistent with the observations in the Raf-1 −/− animals, inactivation of the *c-Raf-1* gene in macrophages by Cre-*lox*P-mediated recombination induces hypersensitivity to pathogen-induced apoptosis, independently of MEK/ERK (Jesenberger *et al.*, 2001).

Several mechanisms have been proposed to explain the antiapoptotic function of Raf-1. It has been suggested that Raf-1 may promote cell survival by antagonizing the proapoptotic apoptosis signal-regulating kinase 1 (ASK1) through a direct inhibitory interaction with the N-terminal domain of ASK1 (Chen *et al.*, 2001). It was also reported that Raf-1 translocates to the mitochondria in response to survival stimuli, where it mounts an antiapoptotic response independently of ERK activation (Wang *et al.*, 1996; Salomoni *et al.*, 1998; Peruzzi *et al.*, 1999; Alavi *et al.*, 2003). It was suggested that, when located to the mitochondria, Raf-1 suppresses cell death by inactivating the proapoptotic Bcl-2 family member Bad (Wang *et al.*, 1996; Salomoni *et al.*, 1998). Phosphorylation of Bad by protein kinases such as Raf-1 or Akt (Wang *et al.*, 1996; Datta *et al.*, 1997) leads to the dissociation of Bad from the prosurvival Bcl-X_L protein, and sequestration of Bad in an inactive complex with 14-3-3 proteins in the cytosol (Zha *et al.*, 1996).

Here, we report that Raf-1 is rapidly cleaved by caspase-9 during apoptosis of hematopoietic cells induced by IL-3 withdrawal. Raf-1 processing cleaves off the N-terminal regulatory domain, leaving an intact C-terminal kinase domain (Raf-1-Ct) that is predominantly localized to the mitochondria. Furthermore, constitutive expression of mitochondria-targeted Raf-1-Ct significantly delays apoptosis of pre-B cells induced by IL-3 withdrawal. These results suggest an important role for caspase-9 mediated cleavage of Raf-1 as a negative feedback regulatory mechanism of hematopoietic cell apoptosis.

Results

Raf-1 is efficiently cleaved in hematopoietic cells undergoing apoptosis through the mitochondrial death pathway

The antiapoptotic potential of Raf-1 led us to evaluate the fate of this protein kinase during apoptosis triggered by the intrinsic (growth factor withdrawal) and extrinsic (death receptor) death pathways. It has been shown that mitochondria play an important role in the early events of the apoptotic process induced by withdrawal of

cytokines in cytokine-dependent hematopoietic cells. Release of cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm and loss of mitochondrial membrane potential have been demonstrated in IL-3-deprived pre-B cells (Vander Heiden *et al.*, 1997; Bojes *et al.*, 1999). In the present study, we used the IL-3 dependent pre-B cell line Ba/F3. Upon IL-3 withdrawal, Ba/F3 cells were arrested in the G1 phase of the cell cycle and died apoptotically, as demonstrated by DNA hypoploidy and proteolytic activation of caspase-3. Ba/F3 cell lysates were prepared at various times after IL-3 withdrawal for immunoblot analysis with anti-Raf-1 antibodies raised against a peptide corresponding to the C-terminus of Raf-1. Within 12 h after IL-3 withdrawal, at which time 10% of the cells were hypoploid, a faint band of ∼40 kDa representing the C-terminal fragment of Raf-1 could be detected (Figure 1a). The abundance of the C-terminal fragment increased with time, concomitantly with a decrease of the full-length 74 kDa Raf-1 protein. In addition to the predominant cleavage product of ∼40 kDa, a less abundant Raf-1-fragment of ∼48 kDa was also observed. As the latter is presumably an intermediate cleavage product, only the generation of the ∼40 kDa fragment was investigated further in detail. A similar Raf-1-specific ∼40 kDa-fragment was also generated during apoptosis induced by IL-3 withdrawal of the IL-3-dependent promyeloid FDCP-1 cell line (Figure 1b). To determine whether proteolysis of Raf-1 also occurs in response to other intrinsic apoptotic stimuli, the Ba/F3 cells were exposed to puromycine (1 μg/ml), an inhibitor of translation elongation. The cell death response and the kinetics of Raf-1-cleavage induced by puromycine were similar to those induced by growth factor withdrawal (Figure 1c).

To determine if Raf-1 is also cleaved upon triggering of the extrinsic death pathway, its cleavage in death receptor-mediated apoptosis was examined in L929sAh-Fas and L929sAhFas.Bcl-2 cells. Stimulation of L929sAhFas cells with agonistic antibodies against Fas induces activation of caspase-8, which, in addition to directly activating executioner caspases, initiates the mitochondrial death pathway via the generation and translocation of tBid to the mitochondria (Denecker *et al.*, 2001). Overexpression of the antiapoptotic Bcl-2 protein in L929sAhFas.Bcl-2 cells specifically inhibits the mitochondrial branch of the Fas pathway, by blocking cytochrome *c* release from the mitochondria (Denecker *et al.*, 2001). Figure 2a shows that in L929sAhFas cells stimulated with anti-Fas, Raf-1 cleavage occurs at a much later stage of the apoptotic response (reflected by the % hypoploid cells) than in Ba/F3 cells deprived of IL-3. Proteolytic activation of executioner caspase-3 in the anti-Fas stimulated L929sAhFas cells was detectable much earlier than the processing of Raf-1 (Figure 2a). Raf-1-cleavage was completely inhibited during the cell death response in anti-Fas stimulated L929sAhFas.Bcl-2 (Figure 2b). All together, these results indicate that cleavage of Raf-1 into the ∼40 kDa C-terminal fragment involves the mitochondria-dependent cell death pathway.
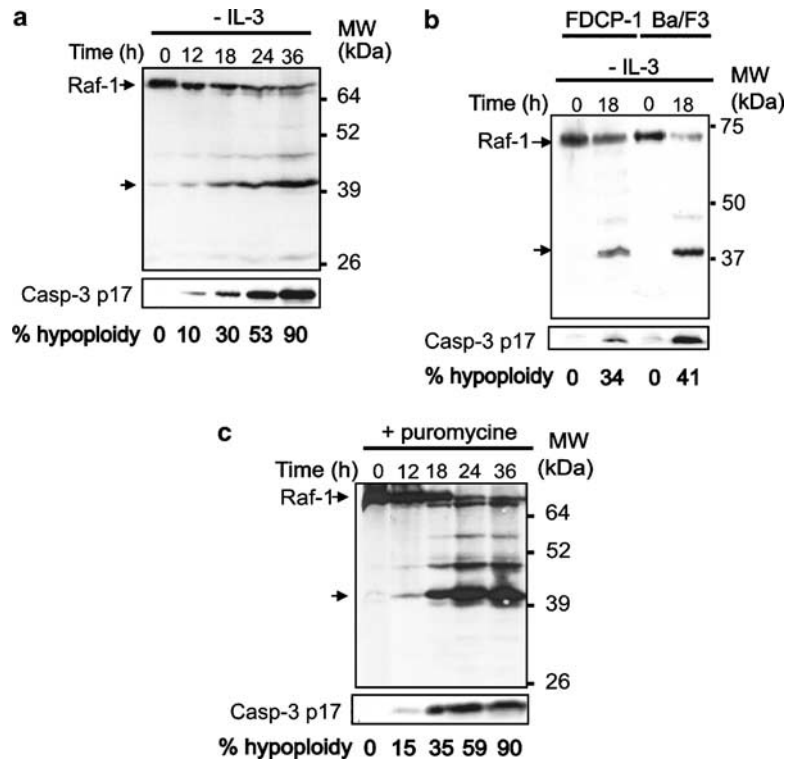
Figure 1 Raf-1 is cleaved following IL-3 withdrawal and puromycine treatment in Ba/F3 cells and FDCP-1 cells. Cleavage of Raf-1 was examined in IL-3 deprived Ba/F3 cells (**a**) and FDCP-1 cells (**b**), as well as in puromycine (1 μg/ml) treated Ba/F3 cells (**c**). At the indicated time points, cell lysates were resolved using 10% SDS–PAGE and subsequently analysed by immunoblotting using a polyclonal anti-Raf C-terminal antibody and an anti caspase-3 antibody. Arrowheads indicate full-length Raf-1 and the ~40 kDa cleavage product. Percentages of hypoploid cells as a measure for apoptosis are indicated at the bottom of each panel

Processing of Raf-1 during apoptosis induced by growth factor withdrawal is mediated by caspase-9

To investigate the potential role of specific caspases in the cleavage of Raf-1, we first attempted to identify the caspases that are proteolytically activated during apoptosis induced by IL-3 withdrawal in Ba/F3 cells (Figure 3a). Processing of procaspase-9 into p35 and p12 subunits and caspase-9 enzymatic activity were detected in the cytosolic extracts as early as 12 h after IL-3 depletion, and coincided with the time of Raf-1 cleavage. The kinetics of proteolytic activation of pro-caspases-3 and -7 were similar to those of pro-caspase-9. In contrast, caspase-8 was cleaved at a later stage. To further investigate which of the activated caspases could be involved in the cleavage of Raf-1, we analysed the effects of several caspase peptide inhibitors (z-VAD.fmk, Ac-DEVD.fmk, z-IETD.fmk), as well as the overexpression of the viral caspase inhibitory protein CrmA or the antiapoptotic protein Bcl-2, on Raf-1 cleavage in Ba/F3 cells. Incubation of Ba/F3 cells with the broad-spectrum caspase inhibitor z-VAD.fmk entirely prevented the cleavage of Raf-1 (Figure 3b, lane 3). On the other hand, Ac-DEVD.fmk and z-IETD.fmk, which display some specificity for caspase-3-like and caspase-8-like caspases, respectively, did not inhibit Raf-1 processing (Figure 3b, lanes 4 and 5). In line with these results, overexpression of the caspase-1 and caspase-8-specific inhibitor CrmA also did not block Raf-1 cleavage in response to IL-3 withdrawal (Figure 3c, lane 4). In contrast, Ba/F3 cells overexpressing Bcl-2 showed no Raf-1 cleavage (Figure 3c, lane 3). All together, these studies indicate that Raf-1 cleavage is dependent on the activation of a z-VAD.fmk-sensitive but Ac-DEVD.fmk- or z-IETD.fmk-insensitive caspase, the activation of which can be inhibited by Bcl-2 overexpression. Taking into account the activation of caspase-9 in IL-3 deprived cells, its z-VAD.fmk sensitivity and its positioning downstream of the mitochondria, caspase-9 is a likely candidate for the Raf-1-cleaving caspase.

To further investigate caspase-9's involvement in Raf-1 processing, *in vitro* translated and ^{35}S-methionine labeled Raf-1 was incubated with purified recombinant active caspase-9, and *in vitro* Raf-1 cleavage was monitored by SDS–polyacrylamide gel electrophoresis. Caspase-9 cleaved Raf-1 into two distinct fragments of ~40 and ~35 kDa (Figure 4a). The size of the higher molecular mass band corresponds to the length of the C-terminal fragment observed in apoptotic Ba/F3 and FDCP-1 cells, further suggesting that caspase-9 is responsible for Raf-1 cleavage in Ba/F3 cells. The 35 kDa product that is generated *in vitro* most likely corresponds to the N-terminal part of Raf-1. Three potential caspase cleavage sites that could result in the generation of fragments with molecular masses
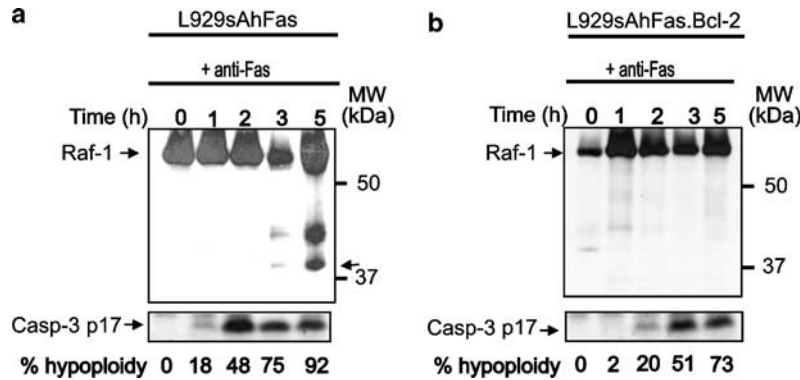
Figure 2 Cleavage of Raf-1 requires activation of the mitochondrial death pathway. (**a**) L929sAhFas cells and (**b**) L929sAhFas.Bcl-2 cells were stimulated with anti-Fas antibodies (100 ng/ml) for the indicated periods of time. Cellular proteins were separated by SDS–PAGE, and the proteolytic processing of Raf-1 and caspase-3 was detected by immunoblotting. Arrowheads indicate full-length Raf-1 and the ~40 kDa cleavage product. Induction of apoptosis was determined by measuring the percentage of hyploid cells

corresponding to those described above were identified in the Raf-1 sequence, at positions D273, D279 and D337 (Figure 4b). To determine which of these sites corresponds to the caspase-9 cleavage site, each Asp residue was mutated to Ala, and the *in vitro* sensitivity of the respective Raf-1 mutants to caspase-9-mediated proteolysis was analysed. Caspase-9-mediated cleavage was completely abolished in the D279A mutant, whereas the D273A and D337A mutants were still processed (Figure 4c). Note that the 40 kDa cleavage product runs slightly slower in the case of the Raf-1 mutants when compared to wild-type Raf-1, which is due to the presence of an E-tag peptide epitope (GAPVPYPD-PLEPRAA) that was fused to the C-terminus of the Raf-1 mutants.

To investigate whether the D279A mutant is protected from cleavage in IL-3 deprived Ba/F3 cells, we stably expressed this mutant (Raf-1E-D279A) and the wild-type Raf protein (Raf-1E) in Ba/F3 cells and analysed their cleavage in IL-3-deprived transfectants (Figure 4d). Whereas the apoptotic responses upon IL-3 withdrawal in the Ba/F3-Raf-1E and Ba/F3-Raf-1E-D279A transfectants are comparable, as revealed by proteolytic activation of caspase-3, processing of the Raf-1E-D279A mutant is suppressed. This result suggests that the D279 caspase-9 cleavage site is also used in dying cells. However, some residual cleavage of the mutant could still be detected after 36 h of IL-3 deprivation, suggesting that in addition to the D279 cleavage site also (an)other cleavage site(s) (is) are (as for instance D273 or D337) maybe involved in Raf-1 processing. All together, the above observations clearly indicate a role for caspase-9 in the cleavage of Raf-1 at position D279 during apoptosis of hematopoietic cells induced by growth factor withdrawal.

As indicated above, the caspase-9 mediated cleavage of Raf-1 separates the N-terminal regulatory domain and the C-terminal kinase domain (Figure 4b). Raf-1 was originally described as an initiator of the MAPK cascade, leading among others to Erk1/2 phosphorylation and the downstream activation of promotors containing a serum responsive element (SRE). To

investigate whether the Raf-1-Ct fragment is still enzymatically active, we ectopically expressed Raf-1, Raf-1-Ct and their respective kinase defective (K375A) mutants in HEK293T cells and determined the phosphorylation state of the Erk1/2 kinase by immunoblotting with phospho-Erk-specific antibodies. In addition, we assessed SRE-mediated gene induction by means of the cotransfected reporter plasmid pSRE-Luc, in which the expression of a luciferase reporter gene is under the control of an SRE (Figure 5). Immunoblotting of total cell lysates revealed that Erk1/2 phosphorylation was enhanced in extracts from cells transfected with either Raf-1 or Raf-1-Ct. However, the increase was most prominent in the Raf-1-Ct expressing lysates, indicating that the cleavage product exerts a stronger kinase activity than the full-length Raf-1 kinase (Figure 5a). In line with this result, SRE-driven luciferase reporter activity was found to be 5- and 2-fold higher in cells expressing Raf-1-Ct and Raf-1, respectively, as compared with that of cells transfected with the kinase defective counterparts (Figure 5b). These results are consistent with previous work showing that the N-terminal domain suppresses the catalytic activity of Raf-1, and its deletion constitutively activates Raf-1 (Stanton *et al.*, 1989; Heidecker *et al.*, 1990).

Raf-1-Ct preferentially localizes to the mitochondria where it can enhance cellular resistance to apoptosis

Raf-1 has been shown to efficiently suppress apoptotic cell death via a process that requires its translocation to the mitochondria (Wang *et al.*, 1996; Salomoni *et al.*, 1998; Majewski *et al.*, 1999). To further investigate the role of caspase-9-mediated cleavage of Raf-1 during apoptosis induced by growth factor withdrawal, we examined Raf-1 expression in mitochondrial and cytosolic fractions of growing and IL-3-deprived Ba/F3 cells. Mitochondrial fractions were prepared by Percoll gradient centrifugation. Purity of the mitochondrial and the cytosolic fractions was judged by Western blot analysis of the mitochondrial marker protein cytochrome oxidase subunit IV (COX IV) and the cytosolic
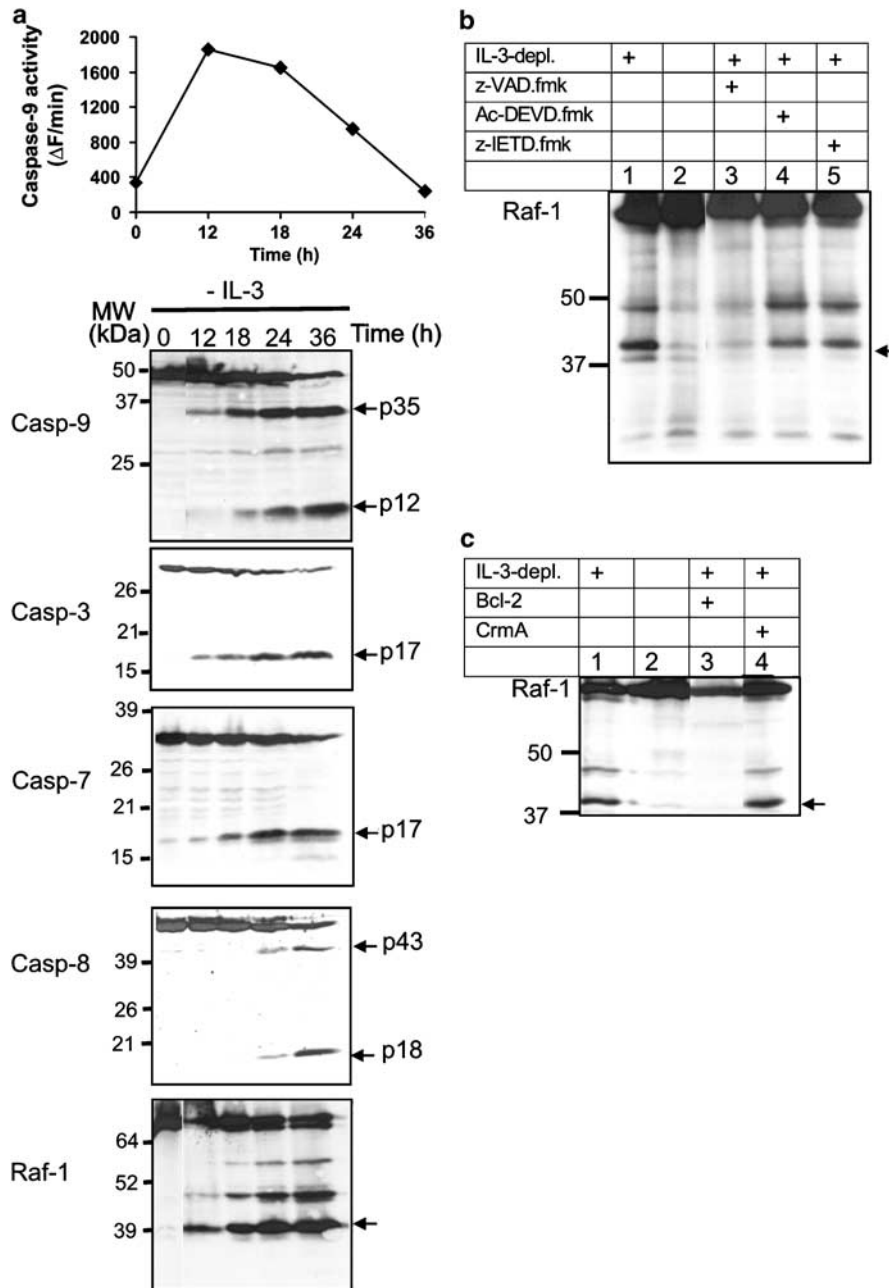
Figure 3 Cleavage of Raf-1 coincides with caspase activation and is blocked by Bcl-2 and z-VAD.fmk. (**a**) Raf-1 cleavage coincides with caspase activation during apoptosis of Ba/F3 cells induced by IL-3 deprivation. Total lysates of cells deprived of IL-3 for the indicated times were assayed for caspase-9 activity using a fluorogenic peptide substrate (Ac-LEHD-amc) as described under 'Material and methods' (upper panel) and resolved using SDS–PAGE for immunoblotting with the polyclonal anti-Raf-1 or anti-caspase antibodies as indicated (lower panels). (**b**) Processing of Raf-1 in the presence of peptide caspase inhibitors. Cells were grown in the presence of IL-3 (lane 2) or deprived of IL-3 for 16 h in the absence (lane 1) or presence of 200μM z-VAD.fmk (lane 3), Ac-DEVD.fmk (lane 4) or z-IETD.fmk (lane 5). Proteolytic processing of Raf-1 was detected by immunoblotting with polyclonal anti Raf-1 antibodies. (**c**) Effects of Bcl-2 and CrmA overexpression on cleavage of Raf-1 induced by IL-3 deprivation. Ba/F3 cells stably overexpressing Bcl-2 (Ba/F3-Bcl-2; lane 3) or CrmA (Ba/F3-CrmA; lane 4) were depleted of IL-3 for 16 h. Stable transfectants which express only the puromycine resistance gene (Ba/F3-puro) served as a control (lanes 1 and 2). Lysates were analysed by immunoblotting with anti-Raf-1 polyclonal antibodies

Erk1/2 MAP kinases, respectively. COX IV was detectable exclusively in the mitochondrial fraction, whereas the Erk1/2 MAP kinases were found mainly in the cytosolic fraction (Figure 6). Immunoblotting with anti-Raf-1 antibodies showed that the great majority of full-length Raf-1 was present in the cytosol of proliferating cells, and only a small part resided in the mitochondria. In contrast, IL-3 depletion resulted in an enrichment of Raf-1 in the mitochondrial fraction. Interestingly, the caspase-9 induced C-terminal
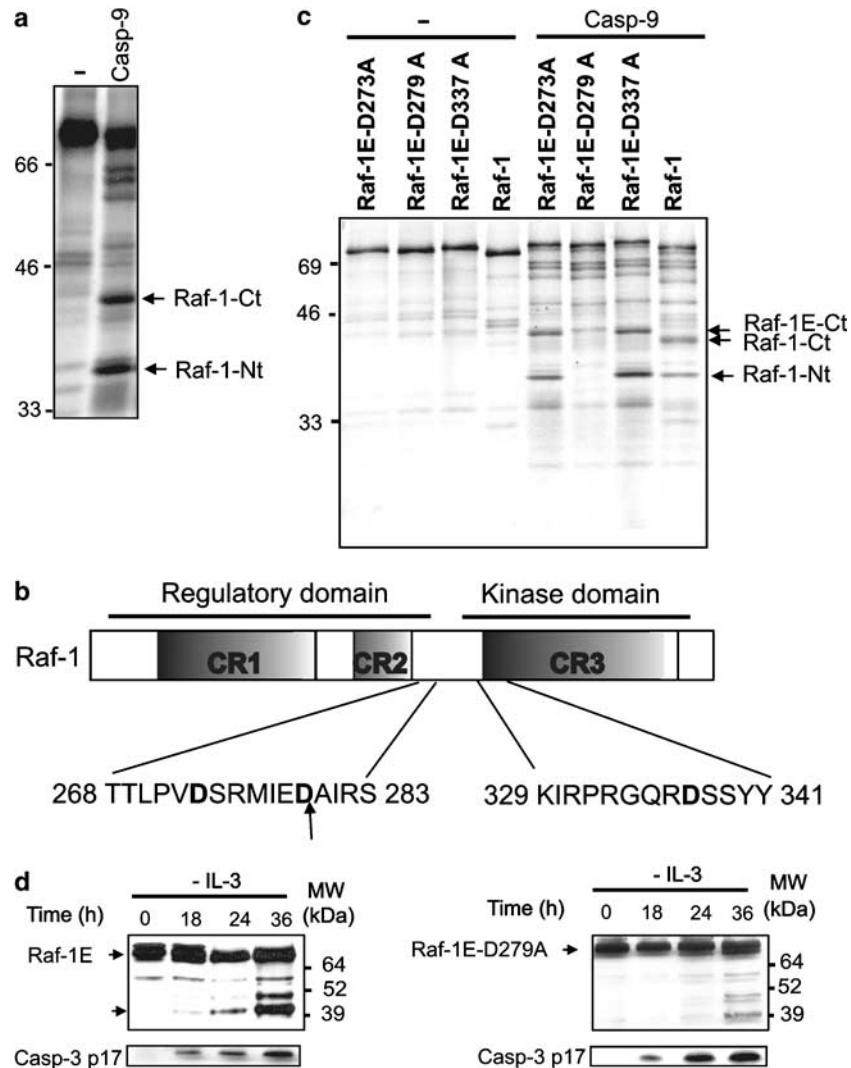
Figure 4 Cleavage of Raf-1 and Raf-1 mutants by caspase-9. (**a**, **c**) *In vitro* cleavage of Raf-1 and Raf-1 mutants by caspase-9. *In vitro* translated and ^{35}S-labeled Raf-1 (**a**, **c**) and E-tagged Raf-1 mutants (Raf-1E) (**c**) were incubated with recombinant caspase-9 for 1 h at 37°C. Reactions were then resolved on 10% (**a**) or 15% (**c**) SDS–polyacrylamide gels followed by autoradiography. Arrowheads indicate bands corresponding to the N-terminal (Nt) and C-terminal (Ct) cleavage products of Raf-1. (**b**) Schematic representation of the primary structure of Raf-1 and the potential cleavage sites for caspase-9. Numbers indicate the position of amino acid residues. CR1, CR2 and CR3 are three regions that are highly conserved among Raf proteins: CR1 is rich in cysteine residues, CR2 is rich in serine/threonine residues, and CR3 is the kinase domain. Aspartic acid residues that were mutated in the present study are indicated in bold. The arrowhead indicates the cleavage site for caspase-9. (**d**) Cleavage of Raf-1 and Raf-1E-D279A during apoptosis of Ba/F3 cells induced by IL-3 deprivation. Total lysates of Ba/F3 cells stably expressing Raf-1E (left panel) or Raf-1E-D279A (right panel), and deprived of IL-3 for the indicated times, were subjected to Western blotting with anti-E tag and anti-caspase-3 antibodies. Arrowheads indicate full-length Raf-1 and the ~ 40 kDa cleavage product

fragment of Raf-1 (Raf-1-Ct) was predominantly present in the mitochondrial fraction (Figure 6).

It has been shown that mitochondrial targeting of a Raf-1 deletion mutant lacking the N-terminal domain (Δ26-303) delays apoptotic cell death in IL-3 deprived 32D.3 cells (Wang *et al.*, 1996). To investigate whether Raf-1-Ct localized in the mitochondria has the same effect on cell survival, we used Ba/F3 cells stably transfected with an expression vector encoding Raf-1-Ct fused with the transmembrane domain of the yeast outer mitochondrial membrane protein Mas70p (M-Raf-1-Ct), for targeting to mitochondria (Hase

et al., 1984). In addition, we also used cells transfected with a similar construct encoding a kinase defective K375A Raf-1-Ct mutant. Immunoblot analysis of lysates prepared from these cells confirmed the expression of all transfected proteins, and confocal microscopy of fixed cells confirmed mitochondrial localization of M-Raf-1-Ct (data not shown). Kinetics of Ba/F3 cell death in response to IL-3 depletion was determined by measuring DNA hypoploidy (Figure 7a), and by assessment of caspase-3 activity (Figure 7b). The latter was determined by measuring the rate of Ac-DEVD-amc cleavage in cell lysates. Ba/F3 cells that were
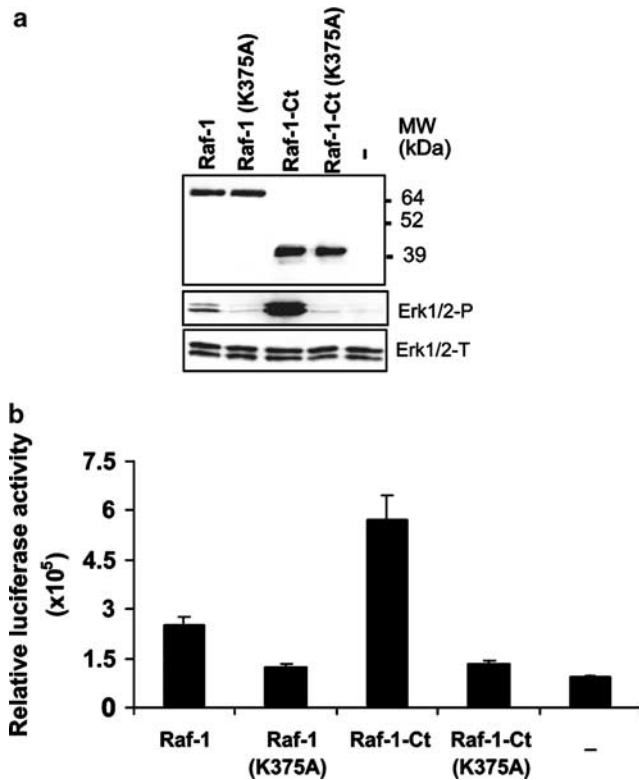
a

b

Figure 5 The caspase-9 induced C-terminal fragment of Raf-1 shows increased activity compared to full-length Raf-1. (**a**) Erk1/2 phosphorylation by Raf-1. Expression vectors encoding the wild-type Raf-1 kinase (Raf-1), the C-terminal cleavage product (Raf-1-Ct) and their kinase defective mutants (Raf-1 (K375A), Raf-1-Ct (K375A)) were transiently transfected in HEK293T cells. After 24 h, cells were lysed and cellular proteins were separated by SDS–PAGE. Expression levels of transfected E-tagged proteins were evaluated by immunoblotting with anti-E-tag antibodies (upper panel). The phosphorylation level of Erk1/2 in the various cell lysates was analysed by immunoblotting with antibodies specific for Thr202/Tyr204 phosphorylated Erk1/2 (Erk1/2-P; middle panel). Total Erk1/2 (Erk1/2-T) levels were revealed by anti-Erk1/2 antibodies (lower panel). (**b**) Induction of SRE-dependent gene expression by Raf-1. The expression vectors encoding the wild-type Raf-1 kinase (Raf-1), the cleavage product (Raf-1-Ct) and their kinase defective mutants (Raf-1 (K375A), Raf-1-Ct (K375A)) were cotransfected with the pSRE-Luc plasmid (Path-Detect, Stratagene), and with the pSV-Sport β-galactosidase plasmid. The latter was used to correct for variation of transfection efficiency. After 24 h, cells were lysed and the activities of luciferase and β-galactosidase were measured. Luciferase activity was normalized to the β-galactosidase activity. Bars represent the average ($n=3$) ± s.d. of normalized luciferase activities. Bars are representative of two independent transfection experiments

transfected only with the plasmid containing the puromycine (puro) resistance gene, and cells that were transfected with Bcl-2 were used as negative and positive controls, respectively. Cell death was significantly delayed in Ba/F3 cells expressing Bcl-2 or M-Raf-1-Ct, but not in those expressing Raf-1-Ct. Moreover, none of the kinase inactive versions of Raf-1, irrespective of their mitochondrial localization, could delay the apoptotic response. These results indicate that mitochondrial localization of the caspase-9-induced Raf-1-Ct fragment prolongs survival of Ba/F3 cells in the absence of IL-3.
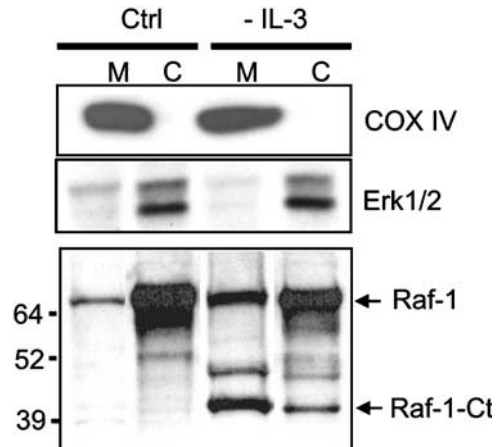

Figure 6 Raf-1-Ct is mainly localized to the mitochondria in IL-3 deprived Ba/F3 cells. Immunoblots of cytosolic fractions (C) and mitochondral fractions (M) of growing (ctrl) and IL-3 deprived (– IL-3) Ba/F3 cells (see 'Material and methods' for details). Equal amounts of protein were loaded in each lane (50 μg) and probed with antibodies against cytochrome oxidase subunit IV (COX IV), Erk1/2, or Raf-1

Discussion

The Raf-1 serine/threonine kinase is an important signal transducing molecule that functions in many growth and developmental pathways. Raf-1 activation is a complex process involving changes in subcellular localization, inter- and intramolecular interactions, and phosphorylation events (reviewed by Chong et al., 2003). In this report we demonstrate that Raf-1 is rapidly processed from its 74 kDa full-length form into a C-terminal fragment of ∼40 kDa (Raf-1-Ct) in the IL-3-dependent Ba/F3 pre-B cell line induced to undergo apoptosis by IL-3 withdrawal. This cleavage requires activation of the mitochondrial death pathway and is mediated by the apical caspase-9 protease. Caspase-9 cleaves Raf-1 at position D279, which is conserved in human and mouse Raf-1. D279 and its context do not fit the proposed consensus motif for a caspase-9 cleavage site (Thornberry et al., 1997; Garcia-Calvo et al., 1999), but the autocatalytic cleavage site (PEPDA) in caspase-9 itself also exhibits poor similarity with the consensus sequence (Stennicke et al., 1999). Previous work has shown that Raf-1 contains an autoinhibitory domain within the first 330 amino acids of its N-terminus (Cutler et al., 1998; Chong and Guan, 2003). Deletions of the N-terminal regulatory domain have been found in several activated forms of Raf genes detected in certain neoplastic human cells (Stanton and Cooper, 1987), resulting in a constitutively active kinase domain that is comparable to the oncogenic v-Raf protein (Cleveland et al., 1994). The mechanism whereby the catalytic domain of c-Raf-1 is released from the autoinhibitory domain is still largely unknown, although a role for phosphorylation of the kinase domain by p21-activated kinase 1 and Src has been demonstrated (Tran and Frost, 2003). Our data indicate that caspase-9 mediated cleavage of Raf-1 at D279, thus separating the
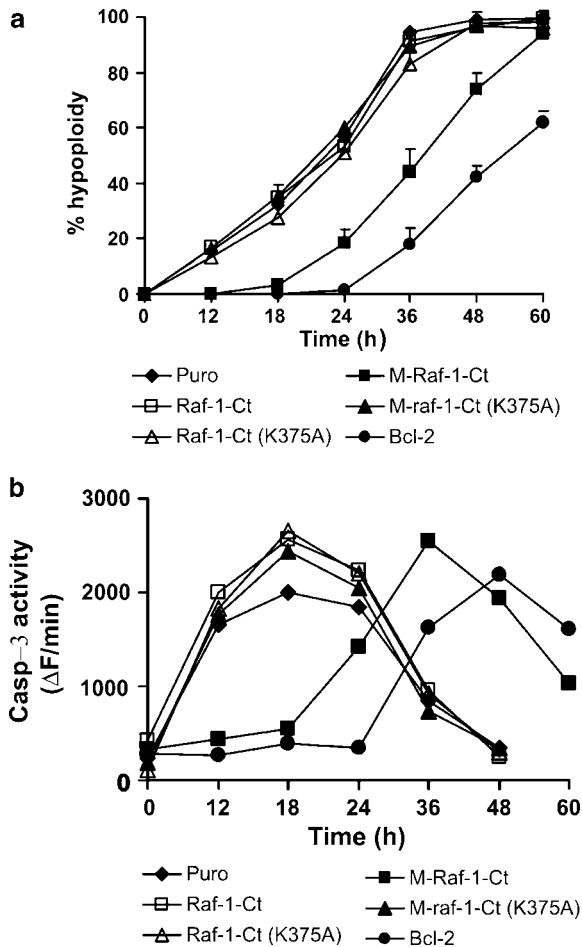
Figure 7 Mitochondrial targeting of Raf-1-Ct enhances Ba/F3 cell survival in the absence of IL-3. Ba/F3 cells stably expressing a nontargeted or a mitochondria-targeted C-terminal Raf-1 fragment (Raf-1-Ct or M-Raf-1-Ct, respectively) or their kinase-defective mutants (Raf-1-Ct-K375A or M-Raf-1-Ct-K375A, respectively) were cultured without IL-3 for the indicated times. Cells transfected with Bcl-2 or only the puromycine resistance gene (Puro) were used as positive and negative control, respectively. (a) The percentage of hypoploid cells, determined by flow cytometry of propidium iodide-stained nuclei, is indicated as a measure for apoptosis. The mean values ($\pm$s.d.) of triplicate cultures are shown. (b) Cell extracts were assayed for caspase-3 activity based on the rate of Ac-DEVD-amc cleavage. Results are representative of three independent experiments

N-terminal regulatory domain from the intact C-terminal kinase domain, might be another mechanism to relieve autoinhibition. It should also be mentioned that relief of autoinhibition is not sufficient for full kinase activation and that this activation still relies on activating phosphorylation mechanisms (Chong and Guan, 2003). A similar mechanism of proteolytic kinase activation by caspases has been demonstrated for a number of other kinases including protein kinase C delta (Emoto *et al.*, 1995) and p21-activated kinase 2 (Bokoch, 1998).

Several lines of evidence indicate an important role for Raf-1 in cell survival signaling (reviewed by Troppmair and Rapp, 2003). However, our knowledge

of the molecular mechanisms by which Raf-1 assures cell survival is still very limited. Evidence for a critical role of mitochondria in Raf-1 survival signaling has been supported by the demonstration of a mitochondrial Raf-1 survival pathway, which involves Bcl-2 driven mitochondrial translocation of the kinase and apoptosis suppression by a mechanism, which leads to the phosphorylation and thereby inactivation of the proapoptotic Bcl-2 family protein Bad (Wang *et al.*, 1996; Salomoni *et al.*, 1998). However, protection by mitochondrial Raf-1 also occurs in cell lacking the expression of Bcl-2 or Bad, implying that Raf-1 may maintain cell survival via Bcl-2/Bad independent pathways. In this context, a role for voltage-dependent anion channel (VDAC) has already been suggested (Le Mellay *et al.*, 2002). So far, we have no clear understanding how Raf-1 gets activated at the mitochondria or even if Raf-1 activation has to occur at the mitochondria. Interestingly, we could demonstrate that the caspase-9-generated Raf-1 C-terminal kinase fragment is predominantly localized in the mitochondrial fraction of IL-3 depleted Ba/F3 cells. The increased expression of full-length Raf-1 in the mitochondria of deprived cells, and several reports on mitochondrial localization of caspase-9 suggest a model in which Raf-1 is translocated to the mitochondria where it is cleaved by caspase-9 (Krajewski *et al.*, 1999; Susin *et al.*, 1999; Costantini *et al.*, 2002; Potokar *et al.*, 2003). However, we were unable to detect caspase-9 in the mitochondrial fraction of IL-3 deprived Ba/F3 cells (van Loo *et al.*, 2002), suggesting that Raf-1 is cleaved by caspase-9 in the cytoplasm and then translocates to the mitchondria. Using fusion of the C-terminal fragment of Raf-1 with a mitochondrial targeting sequence derived from the Mas70p protein, we could show that the caspase-9 generated Raf-1 catalytic fragment needs to localize to the mitochondria in order to fulfill its antiapoptotic function. Since it was shown that the C-terminal half of Raf-1 is sufficient for co-immunoprecipitation with Bcl-2 (Wang *et al.*, 1994), mitochondrial recruitment of the endogenous caspase-9 generated Raf-1 fragment could still be mediated by Bcl-2.

In conclusion, our results suggest an important role for caspase-9 mediated proteolytic activation of Raf-1 in the negative feedback regulation of hematopoietic cell apoptosis induced by growth factor depletion. In certain conditions, for instance during temporary but potentially lethal lack of survival factors, relief of Raf-1 autoinhibition by caspase-9 mediated release of the Raf-1 N-terminal regulatory domain might help to overcome the critical period by blocking the proapoptotic signal at the mitochondria. Refinement of this model remains an area for further study.

Materials and methods

Plasmid constructs

Human Raf-1 cDNA was inserted as an *Eco*R1 fragment into an *Eco*R1-opened pSV-Sport expression plasmid (Invitrogen,

San Diego, CA, USA) to obtain pSV-Sport-Raf-1. Raf-1E-D273A, Raf-1E-D279A, Raf-1E-D337A and Raf-1E-K375A were generated by overlap PCR using pSV-Sport-Raf-1 as a template, and cloned as *Eco*R1/*Not*1 fragments in frame with a C-terminal E-tag into pSV-Sport-E tag (Cornelis *et al.*, 2000). Overlap primers containing the indicated mutations were as follows: Mutant D273A: 5′-CTGCCTGTGGCCAGCAG GATGATTGAGGATGCAATTCGAAGT-3′ and 5′-ACTTC GAATTGCATCCTCAATCATCCTGCTGGCCACAGGCA G-3′; Mutant D279A: 5′-GCTGCCTGTGGACAGCAGGA TGATTGAGGCTGCAATTCGAAG-3′ and 5′-CTTCGAAT TGCAGCCTCAATCATCCTGCTGTCCACAGGCAGC-3′; Mutant D337A: 5′-TTAGGCCTCGTGCACAGAGAGCTT CAAGCTATTATTGG-3′ and 5′-CCAATAATAGCTTGA AGCTCTCTGTGCACGAGGCCTAA-3′; Mutant K375A: 5′-CACGGAGATGTTGCAGTCGCGATCCTAAAGGTTG TCGAC-3′ and 5′-GTCGACAACCTTTAGGATCGCGACT GCAACATCTCCGTG-3′.

The caspase-9 generated Raf-1 C-terminal domain (Raf-1-Ct) and the corresponding kinase inactive mutant Raf-1-Ct-K375A were generated by PCR using, respectively, pSV-Sport-Raf-1E and pSV-Sport-Raf-1E-K375A plasmids as templates, and the primers 5′-CGGGGTACCGCAATTCGAAGTCA CAGCGAATCAGCC-3′ and 5′-TTACCGCTCGAGTCTAT GCGGCACGCGGTTCCAGCGGATC-3′. The obtained *Kpn*1/*Xho*1 Raf-1-specific fragments were subsequently cloned in a *Kpn*1/*Sal*1-opened pCAGGS expression vector.

Mitochondria-targeted Raf-1-Ct (M-Raf-1-Ct) and M-Raf-1-Ct-K375A contain the yeast outer mitochondrial membrane protein Mas70p transmembrane domain (residues 1–30) fused to the N-terminus of Raf-1-Ct and Raf-1-Ct-K375A, respectively. The Mas70p transmembrane domain was obtained by amplifying the corresponding cDNA from yeast genomic DNA with the primers 5′-CCGAGAATTCATGAAGAG CTTCATTACAAGGAACAAGAC-3′ and 5′-CGGGGTAC CTCGTTGTTGTTGTTGTTGCAATTGGTT-3′, and subsequently cloned as an *Eco*R1/*Kpn*1-fragment into pCAGGS-Raf-1-Ct and pCAGGS-Raf-1-Ct-K375A. All the introduced mutations and fusions were verified by sequencing on an ABI373A sequencer (Applied Biosystems, Foster City, CA, USA).

Cell culture and DNA transfection

The IL-3-dependent mouse pre-B cell line Ba/F3 (Palacios and Steinmetz, 1985) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% conditioned medium from WEHI-3B cells as a source of mouse IL-3. Ba/F3 cells were stably transfected by electroporation as described (Cornelis *et al.*, 2000).

The L929sA TNF-sensitive mouse fibrosarcoma cell line was cultured in DMEM with 10% heat-inactivated FCS, 1% glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. L929sAhFas and L929sAhFas.Bcl-2 cells (cl. 6.11) are L929sA cells that stably express the human Fas receptor (Vercammen *et al.*, 1998; Denecker *et al.*, 2001), or the human Fas receptor and the human Bcl-2 gene (Denecker *et al.*, 2001), respectively.

FDCP-1 (Hapel *et al.*, 1984), an IL-3-dependent mouse myeloid progenitor cell line, was grown in RPMI-1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% conditioned medium from WEHI-3B cells as a source of mouse IL-3.

Human embryonic kidney HEK293T cells (a gift from Dr M Hall) were grown in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. HEK293T cells were transiently transfected by the calcium phosphate precipitation method. Transfected cells were lysed in 25 mM Tris phosphate (pH 8), 2 mM DTT, 2 mM CDTA, 10% glycerol, and 1% Triton X-100. Firefly luciferase activity was assayed in a total volume of 30 μl. The reactions were initiated by addition of 15 μl luciferase assay/substrate buffer (40 mM Tricine, 2 mM [MgCO$_3$]4Mg[OH]$_2$, 5 mM MgSO$_4$, 66 mM DTT, 0.2 mM EDTA, 0.5 mM CoA, 1 mM ATP, 1 mM D-luciferin) to 15 μl cell lysate. Light emission was detected by a Topcount scintillation counter. Activity of the β-galactosidase transfection control was measured in a total volume of 200 μl. Cell lysate (20 μl) was added to 160 μl substrate buffer (60 mM Na$_2$PO$_4$, 10 mM KCl, 1 mM β-mercaptoethanol), and the reaction was initiated by adding 20 μl of 50 mM chlorophenolred-β-D-galactopyranoside substrate.

Induction of apoptosis and measurement of DNA hypoploidy

Apoptosis was induced in L929sAFas cells by treatment with 100 ng/ml CH-11 anti-Fas antibody (BioCheck, Burlingame, CA, USA). Apoptosis was induced in Ba/F3 cells by growth factor withdrawal or by treatment with 1 mg/ml puromycine (Sigma, St Louis, MO, USA). In the former case, exponentially growing cells were washed three times with serum-free medium and seeded in DMEM supplemented with 10% FCS, either in the absence or presence of 10% WEHI-3B cell-conditioned medium. The apoptotic response was determined for each stimulus by measuring the amount of hypoploid cells via flow cytometry of propidium iodide stained cells that were permeabilized by freeze-thawing.

Fluorimetric assay for caspase activity

Caspase-3 or -9 activity was measured by incubating 25 μg cell lysate with respectively 50 mM Ac-DEVD-amc or 50 mM Ac-LEHD-amc (Peptide Institute Osaka, Japan) in 150 μl of cell-free system (CFS) buffer (10 mM HEPES–NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH$_2$PO4, 0.5 mM EGTA, 2 mM MgCl$_2$, 5 mM sodium pyruvate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol). The release of fluorescent 7-amino-4-methyl-coumarin was monitored for 1 h at 37°C at 2-min time intervals in a fluorometer (Cytofluor, PerSeptive Biosystems, Cambridge, MA, USA) at an excitation wavelength of 460 nm. Data are expressed as the increase in fluorescence as a function of time (ΔF/min).

In vitro *caspase cleavage assays*

Raf-1, Raf-1E-D273A, Raf-1E-D279A and Raf-1E-D337A in pSV-Sport (1 μg) were used as template for *in vitro* coupled transcription–translation in a reticulocyte lysate system (Promega Biotec, Madison, WI, USA). Translation reactions (2 μl) were incubated with 1 U active recombinant human caspase-9 (MBL, Naka-Ku Nagoya, Japan) in a total volume of 25 μl CFS buffer for 1.5 h at 37°C. In total, 1 U of recombinant caspase-9 is the enzyme activity that cleaves 1 nmol of the caspase substrate LEHD-*p*NA (*p*NA: *p*nitroanilide) per hour at 37°C at saturated substrate concentrations. The resulting cleavage products were analysed by SDS–PAGE and autoradiography.

Isolation of mitochondrial and cytosolic cell fractions

Exponentially growing Ba/F3 cells were washed three times with serum-free medium and seeded in DMEM supplemented with 10% FCS in the absence or presence of 10% WEHI-3B

cell-conditioned medium. Cells were harvested after 20 h, washed three times with buffer A (20 mM MOPS, 1 mg/ml bovine serum albumin, 1 mM EGTA, 100 mM sucrose), and resuspended in buffer B (buffer A plus 100 μg/ml digitonin, 0.1 mM PMSF, 200 U/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 1 mM sodium fluoride). After homogenization (25 strokes with a dounce homogenizer B pestle), samples were centrifuged twice (2500 g, 5 min, 4°C) to remove nuclei, and centrifuged again (10 000 g, 10 min, 4°C) to obtain the heavy membrane pellet. The supernatant was centrifuged again (20 000 g, 10 min, 4°C) and the soluble part was kept as the cytosol fraction. The heavy membrane pellet was resuspended in 2 ml of 15% Percoll (v/v), and loaded on a Percoll gradient (60–40–23%). After centrifugation (32 000 g, 5 min, 4°C), the mitochondrial fraction was isolated and washed twice in CFS buffer. Finally, the pellet was lysed in buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1 mM PMSF, 200 U/ml aprotinin, 10 μg/ml leupeptin, 0.2 mM sodium orthovanadate and 1 mM sodium fluoride. Protein samples (50 μg) of the different subcellular fractions were analysed by Western blotting.

Immunoblot analysis

Cells were washed twice in ice-cold PBS, and equal cell numbers were lysed by direct addition of protein loading buffer (125 mM Tris-HCl pH 6.8, 6% SDS, 1.4 M β-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue) and boiling for 5 min. Cell lysates were separated by SDS–PAGE and transferred onto a nitrocellulose membrane by semidry blotting in a buffer containing 25 mM Tris-HCl pH 8.0, 190 mM glycine and 20% methanol. All further incubations were carried out at room temperature on a platform shaker. Blocking, incubation with antibody and washing of the membrane were done in PBS supplemented with 0.05% Tween 20 (v/v) and 5% (w/v) nonfat dry milk. Primary antibodies used were anti-Raf-1 (Raf-1 (C12), sc133, Santa Cruz, San Diego, CA, USA), anti-mouse caspase-9, anti-Erk1/2 and anti-phospho-Erk1/2 (Cell Signalling Technology, Beverly, MA, USA), and anticytochrome oxidase subunit IV (COX IV) (Molecular Probes, Eugene, OR, USA). Rabbit polyclonal antibodies raised against recombinant murine caspases-3, -7 and -8 were prepared at the centre d'Economie Rurale (Laboratoire d'Hormonologie Animale, Marloie, Belgium). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies against mouse and rabbit immunoglobulin (Amersham Biosciences, Rainham, UK). Immunoreactivity was revealed with the enhanced chemiluminescence method (NEN Renaissance, PerkinElmer Life Sciences Products).

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