

Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis

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Mcl-1 is an antiapoptotic member of the Bcl-2 family that can promote cell viability. We report here that Mcl-1 is a new substrate for caspases during induction of apoptosis. Mcl-1 cleavage occurs after Asp127 and Asp157 and generates four fragments of 24, 19, 17 and 12 kDa in both intact cells and *in vitro*, an effect prevented by selective caspase inhibitors. As a consequence, the resulting protein that lacks the first 127 or 157 amino acids contains only the BH1–BH3 domains of Bcl-2 family members. Mutation of Asp127 and Asp157 abolishes the generation of the 24 and 12 kDa fragments and that of the 19 and 17 kDa fragments, respectively. Interestingly, when expressed in HeLa cells Mcl-1 wt and Mcl-1 Δ 127 showed a markedly different intracellular distribution. Mcl-1 wt colocalized with α -Tubulin near the internal face of the plasma membrane, while Mcl-1 Δ 127 coassociated with Bim-EL at the mitochondrial level. Coimmunoprecipitation experiments also demonstrated that Mcl1 Δ 127 exhibited increased binding to Bim when compared to Mcl-1 wt. Finally, Mcl-1 wt unlike Mcl-1 Δ 127 inhibited Bim-EL-induced caspase activation. Altogether, our findings demonstrate that cleavage of Mcl-1 by caspases modifies its subcellular localization, increases its association with Bim and inhibits its antiapoptotic function.

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Introduction

Myeloid cell leukemia-1 (Mcl-1) was originally identified as a gene upregulated early in the differentiation program of the human myeloid leukemia cell line, ML-1 (Kozopas *et al.*, 1993). Mcl-1 promotes cell viability as do Bcl-2, Bcl-X, and other antiapoptotic

members of this family (Reynolds *et al.*, 1996; Zou *et al.*, 1999; Cory and Adams, 2002). The expression of Mcl-1 appears to be related to the programming of differentiation/development and cell viability/death, and recent data suggest that Mcl-1 may function by providing short-term enhancement of cell viability (Craig, 2002). This is of particular importance in myeloid cells initiating differentiation in response to phorbol esters and in neutrophils undergoing spontaneous apoptosis. Mcl-1 deficiency results in preimplantation lethality and it was very recently reported that mice conditional for Mcl-1 display a profound alteration of lymphoid development (Rinkenberger *et al.*, 2000; Opferman *et al.*, 2003).

Mcl-1 shows sequence similarity, particularly in the carboxyl portion, to Bcl-2 (Kozopas *et al.*, 1993). It is however distinct from Bcl-2 and Bcl-xL in that it lacks a true BH4 domain and is a larger protein that encodes an additional internal PEST domain. Mcl-1 is regulated at the transcriptional, post-transcriptional and translational levels. Induction by phorbol esters in hematopoietic cell lines involved active transcription (Yang *et al.*, 1996; Townsend *et al.*, 1999; Herrant *et al.*, 2002), which is stimulated through the Erk1/2-mediated pathway (Townsend *et al.*, 1998). In other cell systems, the Erk1/2, the p38MAPK, the PI3K/AKT or the JAK/STAT pathways are also involved in Mcl-1 expression (Craig, 2002). Mcl-1 can also be regulated at the post-transcriptional level by alternative splicing (Bae *et al.*, 2000). While full-length Mcl-1 consists of three coding exons, a splice variant, Mcl-1_S arises by the juxtaposition of exons 1 and 3. Full-length Mcl-1 contains the BH1–BH4 Bcl-2 homology domains while Mcl-1_S contains only the BH3 domain. Accordingly as its BH3-only counterparts Mcl-1_S induces cell death rather than promoting cell survival (Bingle *et al.*, 2000). Thus, Mcl-1 can be converted by alternative splicing from an anti to a proapoptotic gene product, as it is the case for Bcl-X_L (Boise *et al.*, 1993).

Finally, Mcl-1 is also modulated post-translationally by phosphorylation (Domina *et al.*, 2000; Jourdan *et al.*, 2000; Inoshita *et al.*, 2002). Two main pathways of Mcl-1 phosphorylation have been reported to date. The first one is stimulated by phorbol esters through Erk1/2 activation and the second one by agents that cause cells

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to accumulate in the G2/M phase of the cell cycle including colchicine, taxol and okadaic acid. Only this later pathway results in a subsequent electrophoretic mobility shift. A fundamental difference between these two pathways is that phorbol ester-induced phosphorylation occurs in viable cells while the taxol and okadaic acid-dependent phosphorylation takes place in cells that are undergoing apoptosis.

Mcl-1 also appears to be rapidly modulated by degradation but whether or not the putative PEST sequence present in the N-terminal part of the molecule is involved in this process is still a matter of debate (Kozopas *et al.*, 1993; Akgul *et al.*, 2000; Breitschopf *et al.*, 2000). Nevertheless, the conclusion emerging from these observations is that Mcl-1 is a highly regulated protein, suggesting that the ability to closely control Mcl-1 gene expression and protein level is critical for the fine tuning of cell fate decisions, more particularly cell death and survival, but also differentiation.

The findings presented in this manuscript describe a new post-translational mechanism for the regulation of Mcl-1 that involves caspase-dependent cleavage. The resulting proteins that contain the BH1–BH3 domains of Bcl-2 family members have an altered antiapoptotic potential but exhibit an increased association with Bim. We concluded that the cleavage of Mcl-1 by caspases during apoptosis generated a dominant-negative molecule with increased affinity for Bim.

Results

Cleavage of Mcl-1 during induction of apoptosis in different cell lines

CH11 induced the rapid cleavage of Mcl-1 in JA3 (Jurkat) cells, which was detectable as soon as 2 h after antibody addition (Figure 1Aa). Interestingly, Mcl-1 was cleaved in several cell lines under various stimuli (Figures 1Ab–d). Mcl-1 cleavage was noticeably observed 4–8 h following induction of apoptosis by staurosporine or actinomycin D in HeLa cells (Figure 1Ab), 18 h after B cell receptor engagement in the immature Ramos B cell line (Figure 1Ac) and 36 h after treatment of the chronic myelogenous leukemia cell line K562 by the Bcr-Abl inhibitor imatinib mesylate (Figure 1Ad). In each case the cleavage of Mcl-1 generated a 24 kDa fragment and was shown to parallel that of Poly-ADP-Ribose-Polymerase (PARP). Interestingly, Mcl-1 cleavage was also detected in primary cells such as thymocytes (Figure 1B) and neutrophils (not shown). When incubated for 4 h at 37°C, mouse thymocytes undergo an apoptotic program that is characterized by DNA fragmentation (Figure 1Ba), caspase activation (Figure 1Bb) and Mcl-1 cleavage (Figure 1Bc). Mouse thymocytes were shown to express the short form of Mcl-1 (Mcl-1_S). Mcl-1_S transcript arises from an alternative splicing that generated an Mcl-1 protein truncated from the middle part of the molecule (Bae *et al.*, 2000). The corresponding 24 kDa short form of Mcl-1 appears to

retain the caspase cleavage site(s) since it gave rise to a 17 kDa fragment in thymocytes undergoing apoptosis (Figure 1Bc).

Mcl-1 cleavage is caspase dependent

Preincubation of JA3, HeLa, Ramos and K562 cells with the pancaspase inhibitor Z-VAD-fmk abolished the generation of the 24 kDa Mcl-1 fragment following CH11, staurosporine, anti-IgM or imatinib mesylate stimulation, respectively (Figure 2A), strongly suggesting that caspases are responsible for the Mcl-1 cleavage in apoptotic cells. We have previously shown that Mcl-1 is a substrate for recombinant caspases *in vitro*, and identified a major Mcl-1 cleavage product as a 24 kDa fragment (Herrant *et al.*, 2002). To analyse more precisely the cleavage of Mcl-1, *in vitro* transcribed and translated ³⁵S-labelled Mcl-1 was incubated at 37°C with either purified recombinant caspases or cellular extracts prepared from Fas-stimulated JA3 cells (Figure 2Ba). The proteolytic profile obtained with recombinant caspases 3, 6 or 7 was indistinguishable from that induced by a cellular extract prepared from Fas-stimulated JA3 cells. Finally, cleavage of Mcl-1 was abolished in the presence of the caspase inhibitors Z-VAD-fmk (not shown) and Ac-DEVD-CHO (Figure 2Bb).

Identification of the caspase cleavage sites in Mcl-1

In view of the results described above, it appears that Mcl-1 is cleaved by caspases at least at two different sites. As the antibody used for the Western blot presented in Figure 1 recognizes the C-terminal domain of the protein, the 24 kDa product most likely corresponds to the C-terminus part of the protein. A careful examination of the Mcl-1 sequence allows us to identify two aspartate residues at position 127 and 157, located in the sequences EELD/G and TSTD/G, respectively, which could match with authentic caspase sites and are compatible with the generation of 24, 19, 17 and 12 kDa fragments. Notably, the EELD/G peptide was strictly conserved in Mcl-1 sequences from different mammalian species (not shown).

To identify formally caspase sites in Mcl-1, we generated point mutations by replacing aspartate 127 (Mcl-1 A127D) or 157 (Mcl-1 A157D) by alanine individually and together in order to obtain the double mutation (Mcl-1 dm). We also realized deletion of the 127 (Mcl-1 Δ127) and 157 (Mcl-1 Δ157) N-terminal amino acids (Figure 3A). Mcl-1 wt, A127D, A157D, dm, Δ127 and Δ157 were next transcribed and translated *in vitro* in the presence of ³⁵S-methionine and incubated with recombinant caspase 3 in either the presence or absence of Ac-DEVD-CHO. As expected, Mcl-1 wt was proteolytically processed in four fragments of 24, 19, 17 and 12 kDa (Figure 3Ba). Mutation of Asp127 was accompanied by the loss of the 24 and 12 kDa fragments (Figure 3Bb), while mutation of Asp157 abrogated the generation of the 19 and 17 kDa fragments (Figure 3Bc). Interestingly, the double-mutant was found to be totally

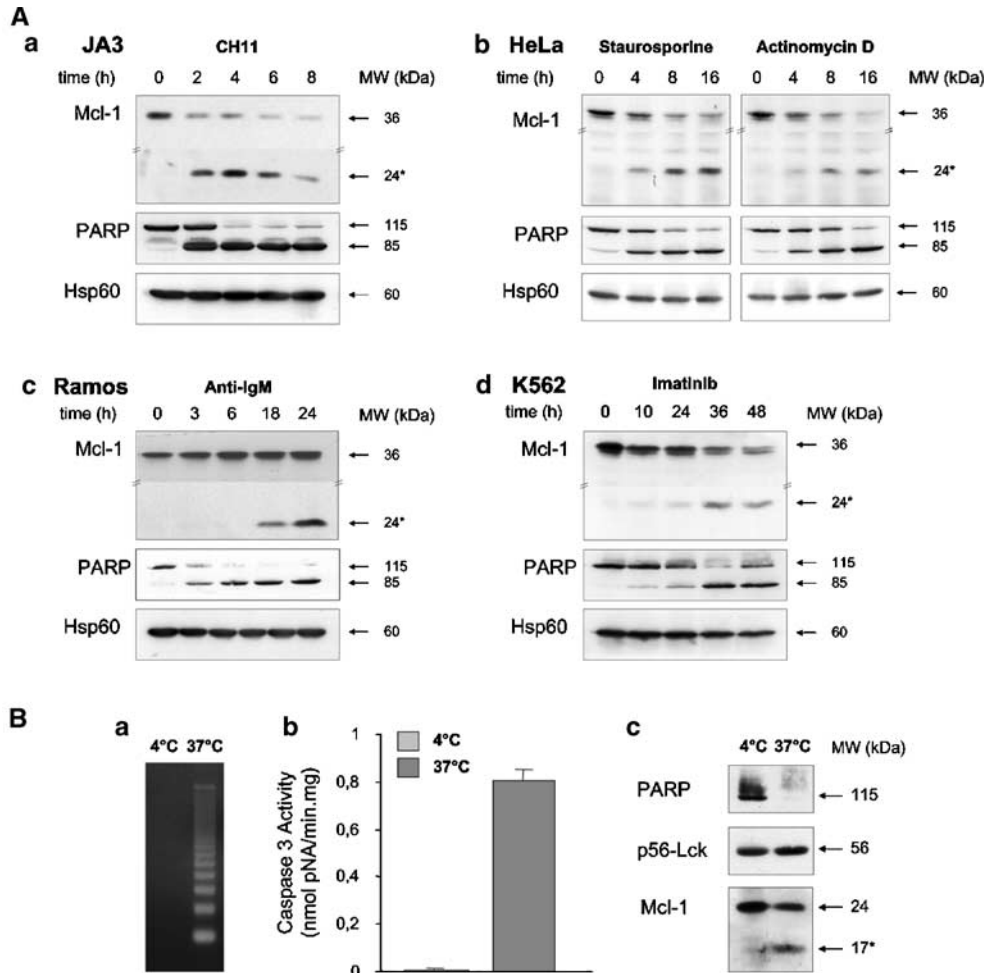


Figure 1 Mcl-1 is cleaved during apoptosis. (A) Mcl-1 is cleaved during apoptosis in different cell lines. (a) JA3, (b) HeLa, (c) Ramos and (d) K562 cells were incubated for different times at 37°C in either the absence or presence of 50 ng/ml CH11, 1 μM staurosporine, 3 μg/ml actinomycin D, 10 μg/ml anti-IgM or 1 μM imatinib mesylate. Cells were lysed and proteins separated by electrophoresis on 12% polyacrylamide gels. Proteins were then transferred onto PVDF membranes, which were incubated with either anti-Mcl-1, anti-PARP or anti-Hsp60 antibodies. * Indicates the cleaved form of Mcl-1. (B) Mcl-1 cleavage in mouse thymocytes undergoing spontaneous apoptosis. Mouse thymocytes were incubated 4 h at 4 or 37°C. (a) Internucleosomal DNA fragmentation was visualised following agarose gel electrophoresis. (b) Caspase activity was assessed on cell lysates using 0.2 mM Ac-DEVD-pNA as substrate. Results are expressed as nanomoles of substrate hydrolysed per min and per milligram of proteins and represent the mean of three different determinations. (c) Cells were lysed and proteins separated by electrophoresis on 12% polyacrylamide gels. Proteins were blotted onto PVDF membranes, which were then incubated with either anti-PARP, anti p56-Lck or anti-Mcl-1 antibodies. * Indicates the cleaved form of Mcl-1

resistant to caspase 3 cleavage (Figure 3Bd). Identical results were obtained using recombinant caspases 6 and 7 (not shown). Deletion of the first 127 amino acids generated the 24 kDa fragment that was cleaved by recombinant caspase 3 after Asp157 in the expected 19 kDa fragment (Figure 3Be). Finally, deletion of the first 157 amino acids gave rise to the 19 kDa fragment that was no more a substrate for recombinant caspases (Figure 3Bf). In each case, Ac-DEVD-CHO was found to abolish Mcl-1 cleavage induced by recombinant caspase 3. On the whole, the experiment shown in Figure 3B allowed us to identify the two caspase cleavage sites present in Mcl-1 as Asp127 and Asp157.

Expression of Mcl-1 in HeLa cells

To verify the expression of the different Mcl-1 constructs, myc-tagged Mcl-1 plasmids were transiently transfected in HeLa cells, and protein lysates were analysed by Western blotting with an anti-Mcl-1 antibody (Figure 4a). myc-Mcl-1 wt and myc-Mcl-1 dm were detected as 50 kDa proteins while Mcl-1 Δ127 was observed as two bands of approximately 36 kDa. Finally, Mcl-1 Δ157 was not detected using the anti-Mcl-1 antibody. As the anti-Mcl-1 antibody used in this study recognizes the middle part of Mcl-1, it is conceivable that deletion of the first 157 amino acids impairs recognition of the protein. The use of an

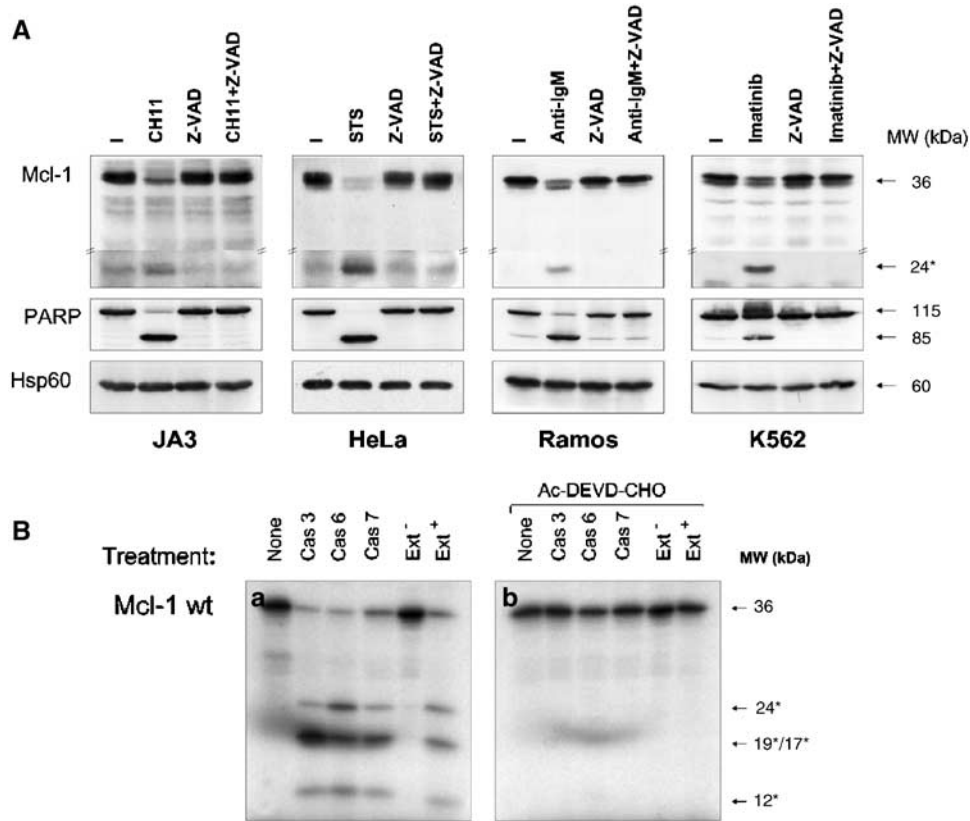


Figure 2 Mcl-1 cleavage is caspase dependent. (A) Effect of Z-VAD-fmk on Mcl-1 cleavage. (a) JA3, (b) HeLa, (c) Ramos and (d) K562 cells were incubated for different times at 37°C in either the absence or the presence of 50 ng/ml CH11, 1 μM staurosporine, 10 μg/ml anti-IgM or 1 μM imatinib mesylate, with or without a 24 h pretreatment with 50 μM Z-VAD-fmk. Then, cells were lysed and proteins separated by electrophoresis on 12% polyacrylamide gels. Proteins were then blotted to PVDF membranes, which were incubated with the mentioned antibodies. * Indicates the cleaved form of Mcl-1. (B) Mcl-1 is substrate for recombinant caspases *in vitro*. Full-length Mcl-1 cDNA was transcribed and translated *in vitro* with ³⁵S-methionine and incubated with purified recombinant caspases 3, 6, 7 (25 ng), Fas-stimulated (Ext⁺) or control (Ext⁻) Jurkat T-cells extracts (100 μg) for 15 h at 37°C in (a) the absence or (b) the presence of Ac-DEVD-CHO (10 μM). The reaction products were then analysed by SDS-PAGE and autoradiography. * Indicates the cleaved forms of Mcl-1

anti-myc antibody confirmed this hypothesis since myc-Mcl-1 Δ157 was readily detected (Figure 4b). When cell lysates prepared from HeLa cells transfected with the different myc-tagged plasmids were incubated for 6 h with recombinant caspase 3, Mcl-1 wt was readily cleaved and the cleavage products were indistinguishable from that obtained in Mcl-1 Δ127 and Mcl-1 Δ157 transfected cells (Figure 4b). As expected, Mcl-1 dm was resistant to caspase 3 cleavage. Finally, as previously shown *in vitro*, myc-Mcl-1 Δ157 was not further cleaved by caspase 3.

Mcl-1 wild type and *Mcl-1* Δ127 exhibited different subcellular localization and degree of interaction with *Bim-EL*

To explore the possibility that caspase-mediated Mcl-1 cleavage is important for regulating cell death, we transiently transfected Mcl-1 wt and Mcl-1 Δ127 constructs into HeLa cells in the presence or absence of an EGFP-*Bim-EL* plasmid. To ensure that transfected HeLa cells do coexpress *Bim-EL* and Mcl-1, we

used five times more Mcl-1 than *Bim-EL* plasmids. In the first set of experiments, we looked for the cellular distribution of the different Mcl-1 proteins as compared to that of EGFP-*Bim-EL*. The use of an anti-myc-TRed antibody revealed that Mcl-1 wt was localized mainly near the internal face of the plasma membrane where it colocalized with α-Tubulin (Figures 5Aa and b). In some cases, we were able to detect a modest association of Mcl-1 wt with *Bim-EL* (Figure 5Ac). By contrast, Mcl-1 Δ127 coassociated closely both with EGFP-*Bim-EL* (Figure 5Ad) and Hexokinase-2, a mitochondrial marker (Figure 5Ae).

To confirm the confocal microscopy results we also performed coimmunoprecipitation experiments. Myc-tagged-Mcl-1 constructs were thus cotransfected or not with EGFP-*Bim-EL* in HeLa cells, as described above. Cellular extracts were then lysed and immunoprecipitated with an anti-*Bim* antibody before Western blotting with an anti-myc antibody. All the Mcl-1 forms were shown to interact with *Bim-EL* (Figure 5B), but a careful examination of Mcl-1 content in total extracts and after immunoprecipitation with anti-*Bim* antibodies

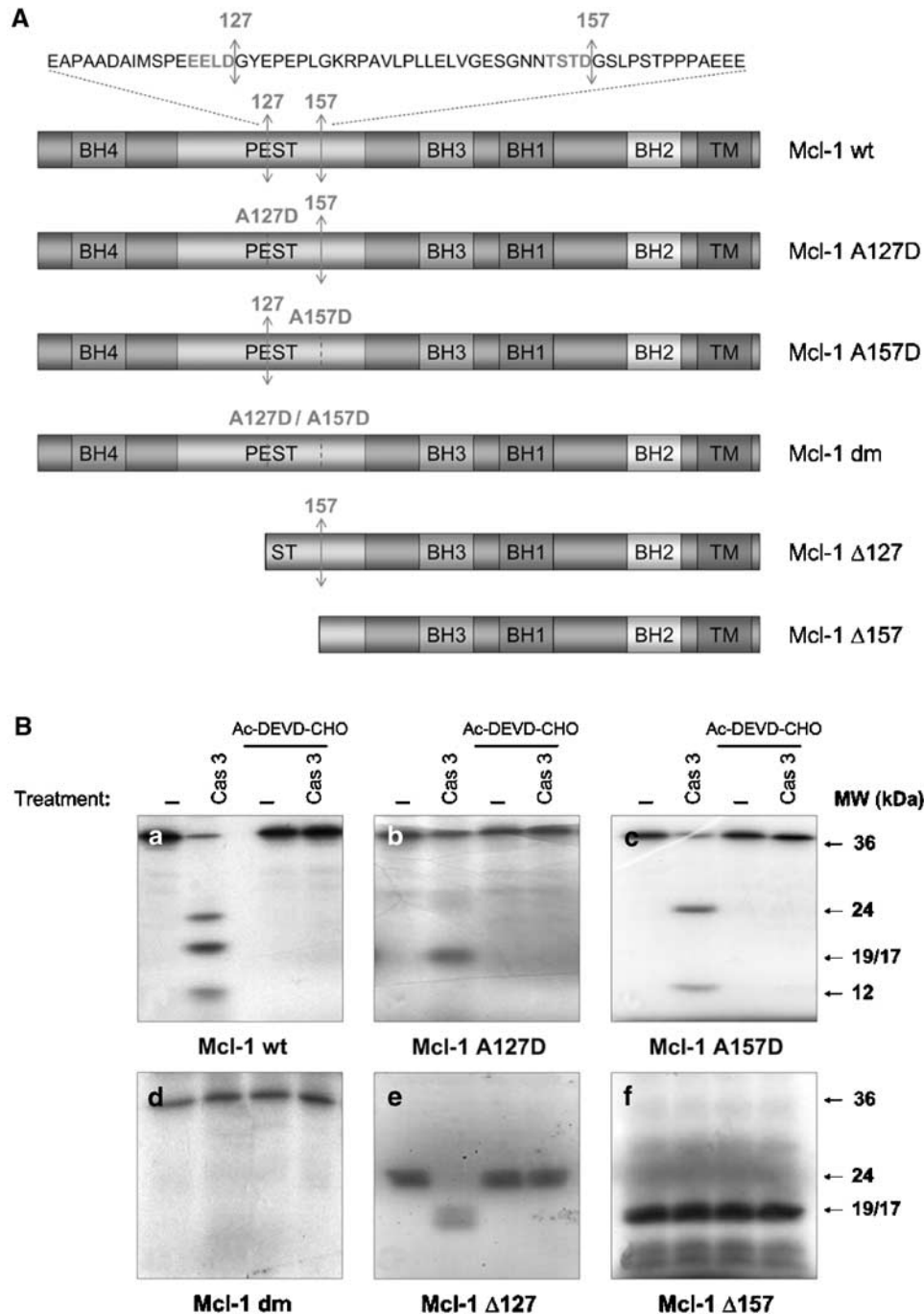


Figure 3 Characterization of the Mcl-1 cleavage sites. (A) Schematic representation of Mcl-1 and mutations. Aspartates 127 and 157 were individually and collectively mutated to alanines by directed mutagenesis. Deleted Mcl-1 Δ127 and Δ157 were obtained. C-terminally myc-tagged plasmids encoding wild-type, mutated and deleted Mcl-1 were constructed. Mutations and deletions were verified by sequencing. (B) Characterization of the Mcl-1 cleavage sites. Wild-type, mutated and deleted Mcl-1 cDNA were transcribed and translated *in vitro* with ³⁵S-methionine and incubated with purified recombinant caspase 3 (25 ng) for 15 h at 37°C in the presence or absence of Ac-DEVD-CHO (10 μM). The reaction products were then analysed by SDS-PAGE and autoradiography

clearly demonstrated that Mcl-1 Δ127 interacted with Bim-EL with a greater efficiency than its Mcl-1 wt or uncleavable counterparts. Indeed, a quantitative analysis of Figure 5B showed that approximately six times more Mcl-1 Δ127 than Mcl-1 wt or Mcl-1 dm associated with Bim-EL (not shown).

Deleted Mcl-1 unlike Mcl-1 wt failed to protect HeLa cells from Bim-EL-induced apoptosis

Owing to the close structural resemblance between Mcl-1 Δ127 or Mcl-1 Δ157 and Bax-like proapoptotic molecules, it was tempting to speculate that cleaved

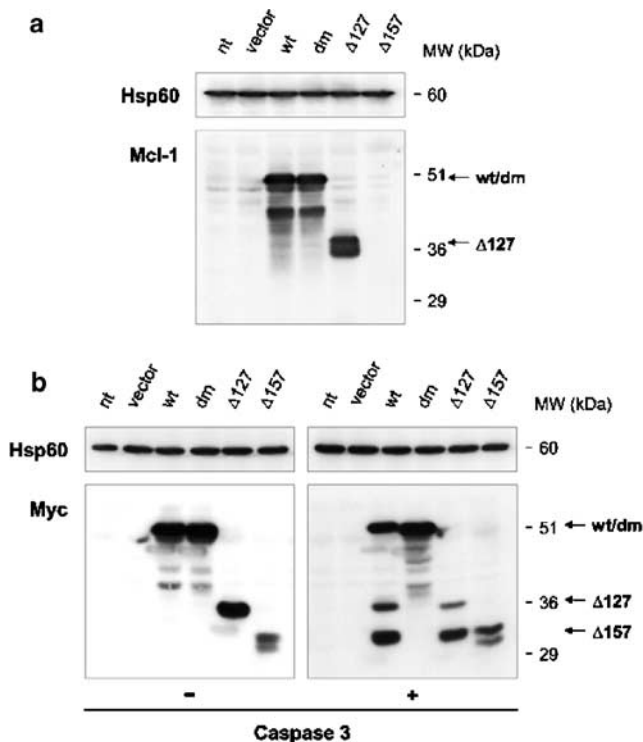


Figure 4 Expression and cleavage of Mcl-1 mutants in HeLa cells. Mcl-1 wt, dm, $\Delta 127$ and $\Delta 157$ were transiently expressed in HeLa cells by using the phosphate Ca^{2+} procedure. (a) Expression of Mcl-1 mutants in HeLa cells. After 48 h, cells were lysed. Proteins were then transferred onto PVDF membranes, which were incubated with either anti-Hsp60 or anti-Mcl-1 antibodies. (b) *In vitro* cleavage of Mcl-1 mutants expressed *in vivo*. After 48 h, cells were lysed. Proteins were or were not incubated with purified recombinant caspase 3 (100 ng) for 15 h at 37°C and transferred onto PVDF membranes, which were incubated with either anti-Hsp60 or anti-Myc antibodies

Mcl-1 might exert a proapoptotic function. However, when expressed in HeLa cells, Mcl-1 $\Delta 127$ and Mcl-1 $\Delta 157$ did not exhibit any proapoptotic function by themselves (Figure 6). We next hypothesized that cleaved Mcl-1 may have altered antiapoptotic function. To confirm this hypothesis, HeLa cells were transfected with Bim-EL plasmids in either the presence or absence of the previously mentioned Mcl-1 constructs. At 24 h following transfection, apoptosis was determined by FACS analysis using an anti-active-caspase-3-PE antibody. Overexpression of Bim-EL in the presence of the control empty vector induced caspase activation in approximately 40% of HeLa cells. As expected, Mcl-1 wt, single-mutated $\Delta 127\text{D}$, $\Delta 157\text{D}$ and double-mutated efficiently inhibited Bim-EL-mediated caspase activation by approximately 70%, while Mcl-1 $\Delta 127$ and Mcl-1 $\Delta 157$ failed to affect Bim-EL-induced caspase 3 activation (Figure 6).

Discussion

We and others have previously identified the cleavage of Mcl-1 in cells undergoing apoptosis (Herrant *et al.*,

2002; Snowden *et al.*, 2003). The present study was thus conducted to better characterize this cleavage, to determine whether caspases participated in this process and finally to clarify its physiological role. We first demonstrated that Mcl-1 is a new substrate for caspases in a broad variety of cell lines and primary cells under a large panel of apoptotic stimuli. Cleavage of Mcl-1 generates four fragments of 24, 19, 17 and 12 kDa, both in intact cells and *in vitro*, and was inhibited by selective caspase inhibitors. Mutation experiments allowed us to determine that Mcl-1 was cleaved after Asp127 and Asp157, in the so-called PEST sequence present in the protein. As a consequence, the resulting protein that lacks the first 127 or 157 amino acids contains only the BH1–BH3 domains of Bcl-2 family members, and thus resembles proapoptotic proteins such as Bax. This observation is in keeping with previous results from Cheng *et al.* (1997) and Fujita *et al.* (1998), showing that Bcl-2 and Bcl-X_L are cleaved by caspase 3 in their N-terminal domains. Thus, cleavage of antiapoptotic Bcl-2 family members during apoptosis could be a more general mechanism than that previously expected. Nevertheless, Mcl-1 cleavage results in the loss of the putative BH4 homology domain that is supposed to be required for the antiapoptotic activity of all the Bcl-2 family members.

Interestingly, the main cleavage site in Mcl-1, that is, EELD/G is closely related to the cleavage sites EEED/G, EERD/G, EEAD/A and EETD/L previously identified in Topoisomerase-1, p59-Fyn, Caspase-2 and SRF, respectively (Li *et al.*, 1997; Earnshaw *et al.*, 1999; Ricci *et al.*, 1999; Bertolotto *et al.*, 2000; Luciano *et al.*, 2001). More interestingly the EELD/G site in Mcl-1 is remarkably conserved in several mammal species whereas it does exist as a significant variation in the second Mcl-1 caspase site (TSTD/G in the human protein), reinforcing the notion that EELD/G represents the physiological caspase cleavage site of the protein. Interestingly, we were unable to detect Bcl-2 cleavage in the different cell lines used in Figure 1 upon conditions where Mcl-1 was readily cleaved. The reasons for this lack of Bcl-2 cleavage are currently unknown, but Fujita *et al.* (1998) reached the same conclusion by comparing Bcl-2 and Bcl-X_L cleavage in CTLL2 cells.

In the present study, we have also shown that deletion by caspase cleavage of the putative BH4 domain and of a part of the PEST sequence generated two Mcl-1 fragments that structurally resemble the proapoptotic protein Bax. Thus, it was tempting to speculate that Mcl-1 $\Delta 127$ and Mcl-1 $\Delta 157$ might kill cells by mechanisms similar to those used by Bax. In fact, despite numerous attempts, we were unable to demonstrate that the cleaved forms generated by caspase cleavage had by themselves a proapoptotic function. Rather, our findings demonstrate that cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-EL-induced apoptosis.

Two Bcl-2 family members have been identified as privileged interactors in the regulation of apoptosis Mcl-1 and the BH3-only protein Bim (Han *et al.*, 2004). Bim activates Bax and Bak by directly occupying the pocket

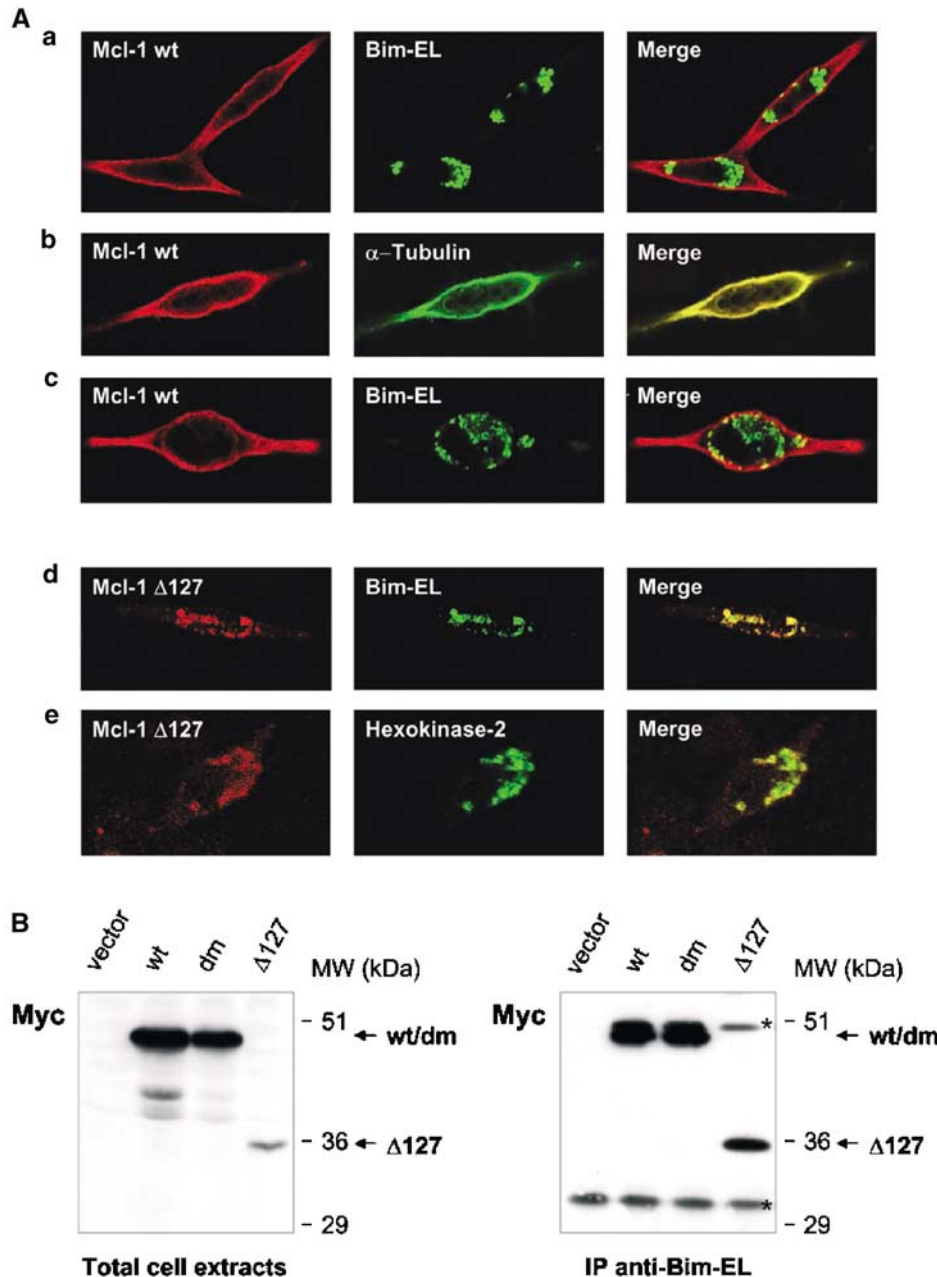


Figure 5 Mcl-1 wt and Mcl-1 $\Delta 127$ exhibited different subcellular localization and degree of interaction with Bim. **(A)** Subcellular localization of Mcl-1 proteins. HeLa cells cultured in $50 \mu\text{M}$ Z-VAD-fmk were transiently transfected with Mcl-1 wt (a, b and c) or Mcl-1 $\Delta 127$ (d and e) in either the presence or absence of EGFP-Bim-EL by using the phosphate Ca^{2+} procedure. After 48 h, cells were fixed in 3.7% formaldehyde, washed in PBS, and permeabilized in 0.2% Triton X-100. Cells were then incubated with anti-Mcl-1 (red), anti- α -Tubulin (green) or anti-Hexokinase-2 (mitochondria) (green) antibodies. Immunofluorescent staining was viewed by confocal microscopy. Z-VAD-fmk did not influence the localization of Mcl-1 or Bim-EL. **(B)** Coimmunoprecipitation experiments. Myc-tagged wt, dm, $\Delta 127$ Mcl-1 and EGFP-Bim-EL were transiently expressed in HeLa cells by using the phosphate Ca^{2+} procedure. After 48 h, cells lysates were incubated with anti-Bim antibody and protein G Sepharose at 4°C overnight. Then beads were washed with lysis buffer and boiled in Laemmli sample buffer before performing SDS-polyacrylamide gel electrophoresis, transfer onto PVDF membrane and immunoblotting with anti-Myc antibody. * indicates the position of the heavy and light chains of Ig

of antiapoptotic Bcl-2 family members such as Bcl-2 and Mcl-1. Recently, recombinant Mcl-1 has been shown to display a strong interaction with the Bim-BH3 domain (Opferman *et al.*, 2003). Our present findings show that in overexpression experiments only a small proportion of Mcl-1 wt (around 5%) coimmunoprecipitates with

Bim. Accordingly, colocalization of Bim with Mcl-1 wt was a relatively rare event even though some coassociation can be detected by confocal microscopy (Figure 5Ac). By contrast, we found that high proportion (20–50%) of Mcl-1 $\Delta 127$ and Mcl-1 $\Delta 157$ (not shown) coimmunoprecipitates with Bim, suggesting that the

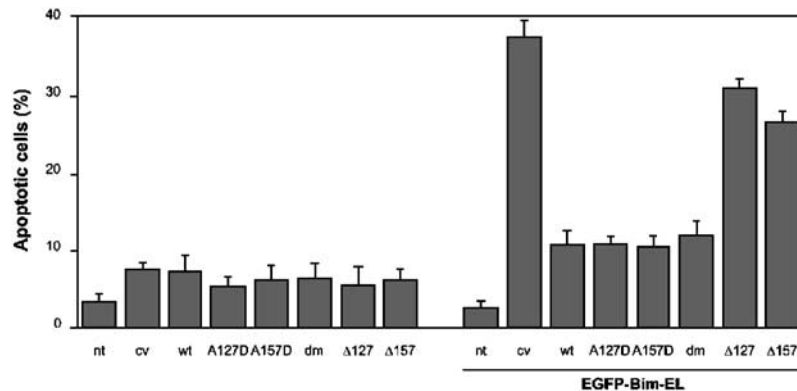


Figure 6 Deleted Mcl-1 unlike Mcl-1 wt failed to inhibit Bim-EL-induced caspase activation in HeLa cells. HeLa cells were transiently transfected with wt, mutated, and deleted Mcl-1 constructs or control vector (5 μ g) in either the absence or presence of EGFP-Bim-EL (1 μ g), using the phosphate Ca^{2+} procedure. At 24 h following transfection, cells were stained using the Anti-active caspase-3-PE MAb Apoptosis Kit. Fluorescence was measured by using the FL1 channel of a FACScan. Data are the mean \pm s.e.m. of three independent experiments

cleaved forms of Mcl-1 display a strong interaction with Bim as compared to Mcl-1. This was confirmed by the nearly complete colocalization of the cleaved form of Mcl-1 with Bim-EL at the mitochondrial level (Figure 5Ae). Bim has been previously shown to be sequestered to the microtubular dynein complex in healthy cells (Puthalakath *et al.*, 1999). Thus, the colocalization of Bim on mitochondria observed in the present study could seem *a priori* unexpected but recent reports from the literature corroborate the constitutive association of Bim on mitochondria or internal membranes in T and B cells (Mouhamad *et al.*, 2004; Zhu *et al.*, 2004). Finally, we found by confocal microscopy that Mcl-1 wt rather than Bim-EL colocalized with α -Tubulin.

Interestingly, it appears that the main characteristics of cleaved Mcl-1 are its very strong association with Bim-EL on the mitochondria as compared to Mcl-1 wt. In light of the present results, it is tempting to propose that the N-terminal domain of Mcl-1 may exert a negative constraint that interferes with its binding to Bim and that caspases by removing this constraint may favour interaction between Bim-EL and cleaved Mcl-1. Finally, Mcl-1 Δ 127 was also found to associate strongly with both Bim-L and Bim-S as compared to Mcl-1 wt (not shown). Thus, cleavage of Mcl-1 by caspases generates a dominant-negative form with an increased ability to interact with Bim. This may give Mcl-1 Δ 127 an advantage with regard to Mcl-1 for Bim binding and may greatly impair any residual protection by uncleaved Mcl-1 in apoptotic cells, thus amplifying cell death. The way by which Mcl-1 Δ 127 achieved this function is not completely understood but the observation that Mcl-1 Δ 127 colocalizes with Bim to mitochondria suggests an effect of cleaved Mcl-1 directly at the Bim level.

Several post-transcriptional and post-translational mechanisms have been reported that modulate the structure and activity of the Mcl-1 protein. First, it has been shown that an exon skipping event in Mcl-1 results in the expression of a BH3-only gene product called Mcl-1_S that promotes cell death (Bingle *et al.*,

2000). Second, phosphorylation by JNK at Ser121 and Thr163 altered Mcl-1 antiapoptotic activity (Inoshita *et al.*, 2002). Finally, a 25 amino acids stretch in the N-terminus of Mcl-1 has been shown to bind with Tankyrase-1, an interaction that could serve to down-modulate specifically this Bcl-2 family member (Bae *et al.*, 2003). It is worth mentioning that both phosphorylation events and Tankyrase-1 binding occurs in the domains of Mcl-1 that are deleted following caspase cleavage.

Mcl-1 was originally identified as a gene induced early by phorbol esters in the process of differentiation of ML1 myeloid leukemia cells (Kozopas *et al.*, 1993). Thus, one possible role of this protein is to function in the fine tuning of differentiation and death processes. Mcl-1 also promotes cell survival in neutrophils and in several hematopoietic malignancies such as multiple myeloma, CLL-B cells and Burkitt's lymphoma (Derenne *et al.*, 2002; Pedersen *et al.*, 2002; Zhang *et al.*, 2002; Jourdan *et al.*, 2003). Noticeably, Mcl-1 is implicated in CLL survival and its overexpression in this disease is associated with poor prognosis (Kaufmann *et al.*, 1998; Kitada *et al.*, 1998). It has been reported that CLL cells undergoing apoptosis show a rapid caspase-dependent cleavage of Mcl-1 (Snowden *et al.*, 2003). Thus, drugs that induced the caspase-dependent cleavage of Mcl-1 may be of potential interest in the treatment of CLL, but also in other hematopoietic malignancies such as multiple myeloma and some lymphoma in which cell survival is intimately linked to Mcl-1 expression (Puthier *et al.*, 2001; Jourdan *et al.*, 2003).

Overall, the present findings demonstrate that caspase cleavage of Mcl-1 is a general mechanism during induction of apoptosis by various stimuli in different cell lines and primary cells. The two resultant fragments exhibit a much higher affinity for Bim than their uncleaved counterparts and are efficiently targeted to mitochondria. Thus, we proposed that cleavage of Mcl-1 by caspases may generate a dominant-negative molecule

that binds Bim with a greater efficiency than Mcl-1 wt, thus counteracting any residual protection by intact Mcl-1.

Materials and methods

Reagents and antibodies

Sodium fluoride, sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, staurosporine and actinomycin D were purchased from Sigma (Saint-Louis, MO, USA). RNase A and proteinase K were from Roche Molecular Biochemicals (Indianapolis, IN, USA). Ac-DEVD-pNA, Ac-DEVD-CHO and Z-VAD-fmk were from Alexis Biochemicals (Lausen, Switzerland). Imatinib mesylate was provided by Novartis Pharma (Basel, Switzerland). Anti-Fas monoclonal antibody (CH11) was from Euromedex (Souffelweyersheim, France) and anti-human IgM was from Jackson Immuno-Research Laboratories (Baltimore, PA, USA). Peroxidase-conjugated, anti-Hsp60, anti-Mcl-1, anti-Myc(9E10), anti-p56-Lck and anti- α -Tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-PARP antibody was from Biomol (Plymouth Meeting, PA, USA). Anti-Hexokinase-2 was from Chemicon International (Temecula, CA, USA) and anti-Bim from Sigma. Anti-mouse and anti-goat antibodies were from Dakopatts (Glostrup, Denmark) and anti-rabbit antibody from Cell Signaling Technology (Beverly, MA, USA).

Cells

Jurkat T cells (clone JA3, a kind gift of Dr John Blenis; Harvard Medical School, Boston, MA, USA) and chronic myelogenous leukemia cells K562 were grown at 37°C under 5% CO₂ in RPMI 1640 medium (Gibco BRL; Paisley, UK) supplemented with 5% FCS and 100 μ g/ml penicillin/streptomycin. HeLa cells were grown at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% FCS and 100 μ g/ml penicillin/streptomycin. The human B lymphoma cell line Ramos (EBV-) has been described elsewhere (Luciano *et al.*, 2003) and was grown at 37°C under 5% CO₂ in RPMI 1640 medium supplemented with 10% FCS, 50 μ M β -mercaptoethanol and 100 μ g/ml penicillin/streptomycin. Freshly prepared thymocytes from 12 weeks mice were plated in RPMI 1640 medium supplemented with 10% FCS at 4 or 37°C under 5% CO₂ as previously described (Pages *et al.*, 1999; Ricci *et al.*, 2001).

Western blot

Cells were incubated with different effectors for the times indicated in the figure legends, and then lysed in buffer B containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 20 mM EDTA, 100 μ M NaF, 10 mM Na₃VO₄, 1 mM PMSF, 1 mM leupeptin, 20 μ g/ml aprotinin, and 1% Triton X-100. A total of 100 μ g of protein were separated on 10–12% polyacrylamide gel and transferred onto PVDF membrane (Immobilon-P, Millipore; Bedford, MA, USA). After blocking nonspecific binding sites, the membranes were incubated with specific antibodies. The membranes were washed three times with TNA-1 % NP-40 (Tris 50 mM, NaCl 150 mM, pH 7.5) incubated further with horseradish peroxidase conjugated antibody for 60 min at room temperature. Immunoblots were revealed by autoradiography using the enhanced chemiluminescence detection kit (Amersham Biosciences; Uppsala, Sweden).

DNA fragmentation

Thymocytes incubated at 4 or 37°C for 4 h were lysed in 200 μ l of lysis buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.2% Triton X-100. Samples were treated with 100 μ g/ml RNase A for 30 min and then treated with 100 μ g/ml proteinase K for 30 min at 37°C as described previously (Herrant *et al.*, 2002). DNA was isopropanol-precipitated, air-dried, resuspended in Tris-EDTA buffer and incubated for 30 min at 55°C. DNA was analysed by electrophoresis on a 1.4% agarose gel containing ethidium bromide.

Caspase activity measurement

Each assay (in triplicate) was performed with 100 μ g of protein prepared from thymocytes incubated at 4 or 37°C for 4 h as described previously (Jacquel *et al.*, 2003). Briefly, cellular extracts were incubated in a 96-well plate with 0.2 mM of Ac-DEVD-pNA as substrate for various times at 37°C. Caspase activity was measured at 410 nm in the presence or absence of 1 μ M of Ac-DEVD-CHO. The specific caspase activity was expressed in nmoles of paranitroaniline released per min and per mg of protein.

Generation of uncleavable or deleted Mcl-1 forms

To obtain the uncleavable forms of Mcl-1, two mutations were introduced in the N-terminal domain that replace aspartates 127 and 157 by alanines (Quickchange site directed mutagenesis kit, Stratagene; La Jolla, CA, USA). The primers used for Mcl-1 Δ 127D were: sens 5' CGCCCGAAGAGGAGCTG GCCGGGTACGAGCCGGAGCC 3', anti-sens 5' GGCTC CGGCTCGTACCCGGCCAGCTCCTCTTCGGGCG 3' and for Mcl-1 Δ 157D sens 5' GGTAATAACACCAGTACGGCC GGGTCACTACCCTCG 3', anti-sens 5' CGAGGGTAGT GACCCGGCCGTACTGGTGTATTACC 3'.

Deleted Mcl-1 were obtained by using the following primers: for Mcl-1 Δ 127: sens 5' CGGCGGCGGCGACTGGCAATG GGGTACGAGCCGGAGCCTCTC 3', anti-sens 5' GCCGC CGCCGCTGACCGTTACCCCATGCTCGGCCTCGGAG AG3', for Mcl-1 Δ 157: sens 5' CGGCGGCGGCGACTGG CAATGGGGTCACTACCCTCGACGCCG 3' and anti-sens 5' GCCGCCGCGCTGACCGTTACCCAGTGATGGGA GCTGCGGC 3'. Mutations and deletions were verified by sequencing. All these cDNAs were cloned into a pcDNA3 myc-his vector (Invitrogen ; Cergy Pontoise, France).

In vitro transcription and translation of Mcl-1

Wild-type, mutated and deleted Mcl-1 constructs were transcribed and translated by using the Promega (Madison, WI, USA) TNT coupled reticulocyte lysate system in the presence of ³⁵S-methionine (ICN Biomedicals; Irvine, CA, USA) as previously described (Bertolotto *et al.*, 2000). Briefly, 2.5 μ l of reticulocyte lysates were incubated in 50 μ l of 25 mM HEPES pH 7.5, 0.1% CHAPS, 5 mM DTT with 25 ng recombinant caspases 3, 6 or 7 (BD PharMingen; San Diego, CA, USA) or 100 μ g of Fas-stimulated Jurkat T cell extracts for 8 h at 37°C. In some experiments, the effect of a caspase inhibitor (Ac-DEVD-CHO, 10 μ M) was also monitored. Proteins contained in cell lysates were electrophoresed on 10% polyacrylamide gels. Gels were autoradiographed using Amersham hyperfilm.

Transfection and cleavage of Mcl-1 mutants in HeLa cells

Myc-tagged wild-type, double-mutated and deleted Mcl-1 constructs were transiently transfected in HeLa cells by using

the phosphate Ca^{2+} procedure. After 48 h, cells were lysed in buffer B. In total, 100 μg of proteins were incubated or not with 100 ng of purified recombinant caspase 3 for 15 h at 37°C. Proteins were electrophoresed on a 10% polyacrylamide gel and transferred onto a PVDF membrane. Wild-type, double-mutated and deleted Mcl-1 proteins were detected by using anti-Mcl-1 or anti-Myc antibodies.

Confocal microscopy

Wild-type and $\Delta 127$ Mcl-1 constructs and/or EGFP-Bim-EL were transiently transfected in HeLa cells by using the phosphate Ca^{2+} procedure. After 48 h, cells were fixed in 3.7% formaldehyde for 20 min at room temperature and permeabilized with 0.2% Triton X-100 for 5 min. Permeabilized cells were incubated with anti-Mcl-1, anti- α -Tubulin or anti-Hexokinase-2 (mitochondria) antibodies for 1 h at room temperature. After three washes with PBS, cells were incubated with the appropriate fluorescent-conjugated secondary antibodies. Cells were then washed three times with PBS and mounted on glass slides in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA). Slides were finally examined and photographed with a confocal microscope (Leica).

Immunoprecipitation experiments

Myc-tagged wild-type, double-mutated or $\Delta 127$ Mcl-1 and EGFP-Bim-EL constructs were transiently transfected in HeLa cells by using the phosphate Ca^{2+} procedure. After 48 h, cells were resuspended in lysis buffer (50 mM Tris-HCl,

pH 7.4, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 0.5% NP-40, 0.1 mM Na_3VO_4 , 1 mM leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM dithiothreitol and 1 mM PMSF). Lysates (500 μl) were then incubated with 1.5 μg of anti-Bim antibody and 15 μl protein G Sepharose (Amersham Biosciences) at 4°C overnight. Beads were next washed five times with 1 ml lysis buffer before boiling in Laemmli sample buffer and performing SDS-polyacrylamide gel electrophoresis, transfer onto nitrocellulose membrane and immunoblotting with the indicated antibodies.

Flow cytometry

HeLa cells were transiently transfected with wild-type, double-mutated, $\Delta 127$ Mcl-1 constructs or control vector (5 μg) and EGFP-Bim-EL (1 μg). After 24 h of expression, cells were stained using the Anti-active-caspase-3-PE MAb Apoptosis Kit (BD Biosciences; San Diego, CA, USA). Fluorescence was measured by using the FL1 channel of a FACScan (Becton Dickinson; Cowley, UK).

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