MCL-1 Inhibits BAX in the Absence of MCL-1/BAX Interaction*

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The BCL-2 family of proteins plays a major role in the control of apoptosis as the primary regulator of mitochondrial permeability. The pro-apoptotic BCL-2 homologues BAX and BAK are activated following the induction of apoptosis and induce cytochrome c release from mitochondria. A second class of BCL-2 homologues, the BH3-only proteins, is required for the activation of BAX and BAK. The activity of both BAX/BAK and BH3-only proteins is opposed by anti-apoptotic BCL-2 homologues such as BCL-2 and MCL-1. Here we show that anti-apoptotic MCL-1 inhibits the function of BAX downstream of its initial activation and translocation to mitochondria. Although MCL-1 interacted with BAX and inhibited its activation, the activity of MCL-1 against BAX was independent of an interaction between the two proteins. However, the anti-apoptotic function of MCL-1 required the presence of BAX. These results suggest that the pro-survival activity of MCL-1 proceeds via inhibition of BAX function at mitochondria, downstream of its activation and translocation to this organelle.

Permeabilization of mitochondria during apoptosis is a major control point for the regulation of this important form of programmed cell death (1). Upon induction of apoptosis, several molecules are released from mitochondria to the cytosol, where they carry their pro-apoptotic functions. One such protein is cytochrome c, which upon its release binds to the adaptor protein APAF-1, leading to its oligomerization and the activation of caspases, the apoptotic proteases (1).

The integrity of the mitochondrial outer membrane is regulated by BCL-2 homologues, a family of proteins that share at least one of the four conserved BCL-2 homology (BH) domains found in BCL-2 (1–3). Three groups of BCL-2 homologues exist based on their BH domains and functions. Anti-apoptotic BCL-2 proteins, such as BCL-2, BCL-XL, and MCL-1, prevent the release of cytochrome c from mitochondria, whereas pro-apoptotic BAX and BAK, which lack the N-terminal BH4, participate in the formation of pores in mitochondria through which cytochrome c is released (1–4). The presence of BAX or BAK is required for cytochrome c to be released and apoptosis to occur (1, 4). The third class of BCL-2 homologues, the BH3-only proteins, contains only the BH3 domain that is required for interaction with the first two groups (1–3, 5).

Two models have been proposed to describe how BH3-only proteins activate the apoptotic machinery (3, 6, 7). The first one posits the existence of two groups of BH3-only proteins: activators such as BID and BIM that directly activate BAX and BAK and derepressors such as Noxa and BAD that bind to anti-apoptotic BCL-2 homologues and thereby prevent them from inactivating both activator BH3-only proteins and BAX/BAK (7, 8). The second model postulates that the main function of anti-apoptotic BCL-2 homologues is to sequester BAX and BAK and prevent them from permeabilizing mitochondria (6, 9). In this model, the role of BH3-only proteins is to bind to anti-apoptotic BCL-2 proteins and prevent them from sequestering BAX and BAK. In this case, the strength of a BH3-only protein is a function of the number of anti-apoptotic BCL-2 proteins it can bind.

In support of the second model, it has been proposed that MCL-1 and BCL-X1 sequester BAX at mitochondria in healthy cells and that inhibition of both MCL-1 and BCL-X1, but not BCL-2, by BH3-only proteins is required to free BAX and induce cytochrome c release (10). This model does not, however, explain the regulation of BAX. In contrast to BAK, which is associated with mitochondria in healthy cells, BAX is monomeric in the cytosol and only translocates to mitochondria following induction of apoptosis. Therefore, BAX is likely to require a specific activation step before it can interact with anti-apoptotic BCL-2 homologues (3, 11).

MCL-1 is unique among anti-apoptotic BCL-2 homologues as it binds a different subset of BH3-only proteins from BCL-2 and BCL-X1 (6, 12). In addition, it has been shown to play an apical role in the inhibition of apoptosis following UV irradiation (13). Although inhibition of BAX by MCL-1 at mitochondria has been suggested to play an important role in its anti-apoptotic function (10), much less is known about the interaction between MCL-1 and BAX. Here we show that MCL-1 inhibits BAX-induced cytochrome c release downstream of BAX conformational change and translocation to mitochondria. The inhibition of BAX by MCL-1 did not require direct interaction

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4 The abbreviations used are: BH, BCL-2 homology; Ad, adenovirus; HM, heavy membrane(s); LM, light membrane(s); MEFs, mouse embryonic fibroblasts; STS, staurosporine; Z, benzoyloxy carbonyl; Fmk, fluoromethyl ketone; HA, hemagglutinin; CHAP5, 3-{[3-cholamidopropyl]dimethylammonio}-1-propanesulfonic acid; GFP, green fluorescent protein; siRNA, small interfering RNA.
between the two proteins, but was nevertheless required for MCL-1 to fulfill its normal anti-apoptotic function.

MATERIALS AND METHODS

Cell Culture, Transfections, and Infections with Adenoviral Vectors—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and 20 mM L-glutamine. To generate cell lines stably expressing MCL-1, HeLa cells were transfected using Lipo-ffectamine 2000 (Invitrogen) and selected with G418. Transient transfections were also carried out using Lipofectamine 2000 and analyzed as indicated in the figure legends. Cotransfections with MCL-1 and BAX, BAK, or BAD were performed using a 2-fold excess of MCL-1 to ensure that cells containing the pro-apoptotic protein also contained MCL-1. For UV irradiation, cells were grown to 95% confluence (or 50% confluency for immunofluorescence), the medium was removed, and the cells were irradiated using 200 ml/cm² UVB light. Fresh medium (containing 50 μM Z-VAD-fmk in the case of immunofluorescence) was then added, and the cells were further incubated for the indicated time. Infections with adenoviral vectors coding for tBID and Noxa (gift of Dr. Gordon Shore) were carried out as described (14, 15) using 50 and 10 plaque-forming units of virus/cell, respectively. All infections were carried in the presence of 50 μM Z-VAD-fmk. Wild-type, BAX knock-out, and MCL-1−/− MEFs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM L-glutamine, 100 mM β-mercaptoethanol, and 0.1 mM nonessential amino acids. MEFs were transfected with Metafectene Pro (Biotex) following the manufacturer’s directions.

Antibodies and Immunoblots—The following antibodies were used: mouse anti-β-actin, mouse anti-FLAG, and mouse anti-HA (Sigma-Aldrich); rabbit anti-MCL-1 and rabbit anti-BAX (Santa Cruz Biotechnology); mouse anti-MCL-1, mouse anti-cytochrome c (78H. 2C12), and mouse anti-calnexin (Pharmingen); rabbit anti-BAK and rabbit anti-p85 phosphatidylinositol 3-kinase (Upstate); mouse anti-BAX (Ab-1) and mouse anti-BAX (Ab-6, clone 6A7) (Oncogene); and rabbit anti-TOM20 (gift from Dr. Gordon Shore (16)). For immunoprecipitation, cells were lysed in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA supplemented with either 1% Triton X-100 or 2% CHAPS, and protease inhibitor mixture (Sigma-Aldrich). Lysates were incubated for 2 h with the antibody and
precipitated with protein A-Sepharose. For immunoblot analysis, proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with specific antibodies. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Amersham Biosciences).

**Fractionation**—Cells were fractionated as described previously (16) with the following modifications. Cells were harvested and resuspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.5, 1 mM EGTA). Cells were broken by passing them 24 times through a 25-gauge needle while being kept on ice. Nuclei and cell debris were removed by centrifugation at 1000 \( \times g \). The resulting supernatant was centrifuged at 9000 \( \times g \) to pellet the HM fraction containing mitochondria. The resulting supernatant was centrifuged at 50000 \( \times g \) for 15 min to generate the LM fraction containing the endoplasmic reticulum (pellet) and the cytosolic fraction (supernatant). For alkaline extraction, 30 \( \mu \)g of HM were incubated for 30 min on ice with 0.1 m \( \text{Na}_2\text{CO}_3 \), followed by centrifugation at 50000 \( \times g \) to recover the membranes (16).

**Immunofluorescence**—For immunofluorescence, cells were grown on coverslips and treated as indicated in the figure legends. Cells were then fixed with 4% paraformaldehyde and analyzed by immunofluorescence. Alexa Fluor 488 and 594 secondary antibodies (Molecular Probes) were used for single-label and double-label immunofluorescence using primary antibodies from different species. Double staining of cells with two mouse primary antibodies (cytochrome c and active BAX (6A7)) was carried out as followed. Cells were first stained with BAX followed by goat anti-mouse Alexa Fluor 594. Cytochrome c antibody was first labeled with Alexa Fluor 488 using a Zenon mouse IgG labeling kit (Molecular Probes) and then incubated with the cells. Coverslips were then visualized using an Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc.) with a 100\( \times \)1.30 oil objective (Carl Zeiss Microimaging, Inc.). Images were captured and overlaid using Northern Eclipse software. A minimum of four fields were counted per coverslip, for a total of at least 100 cells. Results were expressed as the number of cells positive for the marker (cytochrome c release, active BAX, and active BAK) over the total number of cells counted. All transient transfections were analyzed by costaining with an MCL-1 antibody, whereas activation of BAX and BAK was analyzed using N-terminal epitope-specific antibodies for BAX (6A7) and BAK (Ab-1). For MCL-1 (high) cells, only transfected cells (identified by costaining with an MCL-1 antibody) were scored. Shown are the results of three independent experiments ± S.D., \( * \), \( p < 0.01; ** \), \( p < 0.05 \). C, HeLa cells stably expressing either low levels of MCL-1 or vector alone were treated with 200 ml/cm\(^2\) UV light (A) or 50 plaque-forming units of Ad tBID/cell (B) in the presence of 50 \( \mu \)M Z-VAD-fmk; fixed after 4 and 8 h, respectively; and analyzed by immunofluorescence. Cytochrome c (Cyt c) release was scored using a cytochrome c-specific antibody, whereas activation of BAX and BAK was analyzed using N-terminal epitope-specific antibodies for BAX (6A7) and BAK (Ab-1). For MCL-1 (high) cells, only transfected cells (identified by costaining with an MCL-1 antibody) were scored. Shown are the results of three independent experiments ± S.D., \( * \), \( p < 0.01; ** \), \( p < 0.05 \). C, HeLa cells stably expressing either low levels of MCL-1 or vector alone were treated with 200 ml/cm\(^2\) UV light in the presence of 50 \( \mu \)M Z-VAD-fmk and fixed after 4 h. Cells were then stained using antibodies against cytochrome c and active BAX (6A7) and analyzed by immunofluorescence. Representative images are shown. Ctrl, control.

**RESULTS**

**MCL-1 Inhibits BAX- and BAK-dependent Cytochrome c Release**—To gain insight into the potential regulation of BAX and BAK by MCL-1, we generated HeLa cells expressing different amounts of MCL-1. Although high MCL-1 expression levels could be achieved by transient transfection (4.8 ± 0.4-fold, 200 10/300 GL column (Amersham Biosciences) at a flow rate of 0.5 ml/min. 0.5-ml fractions were collected for analysis. Calibration standards (Amersham Biosciences) were run under identical conditions to determine the elution profile of the column.

**Gel Filtration**—Cells were grown to confluency in 2 \( \times \)150 mm dishes; treated with 200 ml/cm\(^2\) UV light; collected after 4 h; and resuspended in 500 ml of 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 2% CHAPS. After centrifugation for 15 min at 50000 \( \times g \), the supernatant was loaded onto a Superdex 200 10/300 GL column (Amersham Biosciences) at a flow rate of 0.5 ml/min. 0.5-ml fractions were collected for analysis. Calibration standards (Amersham Biosciences) were run under identical conditions to determine the elution profile of the column.

**RESULTS**

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MCL-1 Inhibits BAX Downstream of Its Translocation to Mitochondria—Concomitant with its conformational change, BAX translocates from the cytosol to mitochondria, where it undergoes upon induction of apoptosis. The effect of MCL-1 on the activation of endogenous BAX and BAK, as well as on cytochrome c release, was thus tested in a context where apoptosis induction depends on these upstream activators. Expression of MCL-1 decreased in a dose-dependent manner the number of cells with cytochrome c release after either UV treatment (Fig. 2A) or infection with an adenovirus expressing the activated form of the BH3-only protein BID (Ad tBID) (Fig. 2B). Similarly, MCL-1 caused a dose-dependent inhibition of BAK conformational change (Fig. 2, A and B). In contrast, whereas high expression of MCL-1 did prevent BAX activation (6A7-positive), a lower concentration of MCL-1 had no effect (Fig. 2, A and B). This resulted in the appearance of BAX (6A7-positive) cells in which cytochrome c had not been released (Fig. 2C, arrows), indicating that MCL-1 can block cytochrome c release without affecting BAX initial conformational change. As MCL-1 could nevertheless block BAX-dependent cytochrome c release (Fig. 1), these results suggest that MCL-1 could inhibit BAX downstream of its conformational change.

MCL-1 Inhibits BAX Downstream of Its Translocation to Mitochondria—Concomitant with its conformational change, BAX translocates from the cytosol to mitochondria, where it inserts in the outer membrane and becomes resistant to alkaline extraction (3, 11). To test whether MCL-1 affects the translocation of BAX to mitochondria, HeLa cells expressing the activated form of the BH3-only protein BID (Ad tBID) (Fig. 2B). Similarly, MCL-1 caused a dose-dependent inhibition of BAK conformational change (Fig. 2, A and B). In contrast, whereas high expression of MCL-1 did prevent BAX activation (6A7-positive), a lower concentration of MCL-1 had no effect (Fig. 2, A and B). This resulted in the appearance of BAX (6A7-positive) cells in which cytochrome c had not been released (Fig. 2C, arrows), indicating that MCL-1 can block cytochrome c release without affecting BAX initial conformational change. As MCL-1 could nevertheless block BAX-dependent cytochrome c release (Fig. 1), these results suggest that MCL-1 could inhibit BAX downstream of its conformational change.

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light, whereas the amount of BAK in HM did not change. This suggests that MCL-1 blocks BAX downstream of its mitochondrial translocation.

Following its translocation to mitochondria, BAX forms large oligomers that have been associated with cytochrome c release (3, 11, 20). Formation of BAX oligomers was analyzed using the chemical cross-linker bismaleimidohexane. Whereas in untreated cells BAX remained as a 25-kDa monomer following cross-linking with bismaleimidohexane, UV treatment caused the appearance of higher molecular mass bands consistent with the formation of BAX dimers as well as larger oligomers (Fig. 3B). The presence of low amounts of MCL-1 almost completely inhibited the formation of high molecular mass BAX oligomers (Fig. 3B, *), although there was only a small reduction in BAX dimers (**).

To further investigate the possibility that MCL-1 could inhibit the formation of high molecular mass BAX complexes, a second approach was used to assess BAX oligomerization. On a gel filtration column, BAX migrated as a 25-kDa monomer in control cells (Fig. 3C). However, upon induction of apoptosis by UV radiation, it could be detected in complexes ranging in size from 50 to >500 kDa, reflecting the oligomerization of BAX (Fig. 3C). Strikingly, UV irradiation in MCL-1 (low) cells induced the formation of only small BAX complexes (60–150 kDa) (Fig. 3C), whereas higher expression of MCL-1 (achieved by treating the cells with the proteasome inhibitor MG132 to prevent MCL-1 degradation) completely blocked the formation of BAX-containing complexes. Altogether, these experiments indicate that MCL-1 inhibits the formation of large BAX oligomers.

MCL-1 Inhibits BAX in the Absence of Interactions between the Two Proteins—One possible explanation for the results presented in Fig. 3 is that active BAX (6A7-positive) is sequestered in a BAX-MCL-1 complex. To investigate the interactions between BAX, BAK, and MCL-1, we first determined the subcellular localization of the endogenous proteins. Control HeLa cells were fractionated into cytosolic (S100), HM, and LM fractions and analyzed by Western blotting for the presence of the indicated BCL-2 homologues as well as the mitochondrial marker TOM20 and the endoplasmic reticulum protein calnexin. A, MCL-1 associates with BAX but not BAX. HeLa cells were solubilized in buffer containing CHAPS (C) or Triton X-100 (T) and immunoprecipitated (IP) with an antibody against MCL-1. Interactions were analyzed by Western blotting using antibodies against BAX, BAK, and MCL-1. B, MCL-1 transiently transfected with MCL-1 were either left untreated or infected with 50 plaque-forming units of Ad tBID/cell for 8 h. Cells were then fixed and stained using antibodies against MCL-1 (Alexa Fluor 594; red) and the mitochondrial marker TOM20 (Alexa Fluor 488; green). Ctrl, control, D, HeLa cells stably expressing MCL-1 (low) were infected with 50 plaque-forming units of Ad tBID/cell for 8 h. Cells were then fixed and stained using antibodies against MCL-1 (Alexa Fluor 594; red) and BAX (6A7; Alexa Fluor 488; green). Representative images are shown.

21), also suggests that they might not interact under apoptotic conditions.

Subcellular localization of BAX and MCL-1 in apoptotic cells was studied by immunofluorescence. In HeLa cells transiently expressing MCL-1, MCL-1 colocalized with the mitochondrial marker TOM20 (Fig. 4C). Upon induction of apoptosis with Ad tBID, the mitochondrial network became extensively fragmented, as a consequence of DRP1-dependent mitochondrial fission (22). However, MCL-1 still colocalized with TOM20 (Fig. 4C). Upon induction of apoptosis, BAX has been shown to form clusters that localize to mitochondrial fission sites (23). These clusters were readily observed upon Ad tBID treatment.
in HeLa cells stably expressing MCL-1, conditions that do not prevent the conformational change in BAX (Fig. 2B). However, whereas BAX clusters were clearly adjacent to MCL-1 staining, the two proteins did not colocalize (Fig. 4D). Furthermore, MCL-1 remained in a 200-kDa complex irrespective of apoptosis induction and only partially cofractionated with BAX in UV light-treated cells (Fig. 5A). Altogether, these results suggest that MCL-1 and BAX do not directly interact in cells.

Inhibition of Apoptosis by MCL-1 Is Dependent on BAD-inhibited BCL-2 Homologues—Several proteins have been suggested to regulate BAX activation and function during apoptosis (3). Both DRP1 and Bif1 have been proposed to be important for BAX to induce cytochrome c release (23, 24). However, both proteins eluted from the gel filtration column in complexes that are distinct from MCL-1, in control as well as in apoptotic cells (Fig. 5A). This is consistent with our inability to immunoprecipitate either of these proteins with MCL-1 (data not shown). In addition, the elution profiles of Bif1 and DRP1 were not affected by the presence of MCL-1 (data not shown).

On the other hand, the anti-apoptotic BCL-2 homologue BCL-XL is known to bind to the active conformer of BAX at the mitochondria and inhibit its function. Indeed, BCL-XL co-eluted with BAX in UV light-treated MCL-1 (low) cells (Fig. 5A), suggesting that BAX might be associated with BCL-XL in these cells.

To further test the involvement of anti-apoptotic BCL-2 homologues in the function of MCL-1, we took advantage of the differences in the specificity of BH3-only proteins for anti-apoptotic BCL-2 homologues (6, 12). We first validated the approach by inhibiting MCL-1 using Noxa, a BH3-only protein that targets MCL-1, but not BCL-2 or BCL-XL (6, 12). Ad Noxa sensitized HeLa cells to UV light-induced cytochrome c release (14) and also inhibited the function of low levels of MCL-1 over Noxa in this case. These results suggest that the anti-apoptotic effect observed in MCL-1 (low) cells is the direct consequence of the increased MCL-1 expression.

The BH3-only protein BAD targets BCL-2, BCL-XL, and BCL-W, but not MCL-1 or A1 (6, 12), allowing the specific inhibition of BCL-XL-like proteins as opposed to MCL-1. HeLa cells expressing increasing amounts of MCL-1 were transfected with BAD under conditions causing minimal cytochrome c release and treated with UV light. Transfected cells were then...
Inhibition of Apoptosis by MCL-1 Depends on the Presence of BAD—The results presented above suggest that MCL-1 inhibits the anti-apoptotic function of low levels of MCL-1, but not high levels (Fig. 5B). As BAD does not interact with MCL-1 (6, 12), these results suggest that inhibition of BAX by MCL-1 depends on the function of other BAD-sensitive, anti-apoptotic BCL-2 homologue(s) such as BCL-XL.

Inhibition of Apoptosis by MCL-1 Depends on the Presence of BAX—The results presented above suggest that MCL-1 blocks the function of BAX downstream of its initial activation and in the absence of a direct interaction between the two proteins. Several approaches were used to assess the importance of BAX for the anti-apoptotic activity of MCL-1. BAX and BAK were first knocked down using siRNA in HeLa cells stably expressing either MCL-1 (low expression) or vector alone. BAX siRNA reduced BAX levels by 60%, whereas BAX siRNA decreased BAX expression by 90% compared with treatment with GFP siRNA (Fig. 6A). Knocking down either pro-apoptotic protein significantly reduced UV light-induced cytochrome c release (Fig. 6B), indicating that both BAX and BAK are important for UV light-induced apoptosis. Interestingly, whereas MCL-1 still conferred protection when BAX was knocked down, it had no effect on cells lacking BAX (Fig. 6B), suggesting that the presence of BAX is required for MCL-1 to exert its anti-apoptotic activity. Similarly, whereas overexpression of MCL-1 in wild-type MEFs inhibited staurosporine (STS)-induced cytochrome c release, it had no effect on BAX−/− MEFs (Fig. 6C). The lack of effect of MCL-1 in BAX−/− MEFs was not the consequence of reduced MCL-1 expression because MCL-1 was expressed at least as much in BAX−/− MEFs as it was in wild-type MEFs (Fig. 6D).

The inhibition of BAX and BAK by MCL-1 was further addressed using MCL-1 knock-out cells (MCL-1−/−). Wild-type and MCL-1−/− MEFs were treated with STS and analyzed by immunofluorescence for the presence of activated BAX and BAK. STS treatment resulted in the appearance of active BAK in both wild-type and MCL-1−/− cells, although in a higher proportion of the latter (Fig. 7A; quantification in B). In contrast, whereas punctate BAX staining, indicative of its activated state, was readily detectable in MCL-1−/− cells, few positive wild-type cells could be detected (Fig. 7A and B). As BAX and BAK activation were similar in MCL-1−/− MEFs (Fig. 7B), these results suggest that the presence of MCL-1 has a greater effect on BAX than on BAK. Similarly, whereas transfection of MCL-1−/− cells with HA-BAX or FLAG-BAK caused comparable cytochrome c release, the wild-type cells were more resistant to HA-BAX than to FLAG-BAK (Fig. 7C). Expression levels of HA-BAX and FLAG-BAK were similar, however (Fig. 7D). Together, these results suggest that the presence of MCL-1 provides a greater inhibition of BAX than of BAK.

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DISCUSSION

BAX activation is dependent on the function of BH3-only proteins, although their mechanism of action is still debated. Contrary to BAK, which could conceivably be constitutively active but kept in check by proteins at the surface of mitochondria (10), BAX is a cytosolic monomer in control cells and thus requires a signal for its activation (3, 20). Indeed, tBID or peptides corresponding to its BH3 domain can activate BAX in vitro (3, 7, 25). BAX activation is a multistep process involving several changes in the structure of the protein (11). BAX must first change its conformation (becoming 6A7-positive) and translocate to mitochondria, where it becomes inserted in the outer membrane. The active conformer of BAX can then oligomerize to cause the release of cytochrome c from mitochondria. Anti-apoptotic BCL-2 homologues such as BCL-XL can bind to this activated conformer and inhibit further oligomerization (3, 18).

Previous studies have shown that MCL-1 interacts with BAK and inhibits its function (10, 26). Accordingly, MCL-1 inhibited BAK conformational change in a dose-dependent manner in HeLa cells. On the other hand, the relationship between MCL-1 and BAX has been less clear, especially with regards to the potential interactions between the two proteins (discussed in Ref. 3). MCL-1 has been shown previously to inhibit BAX translocation to mitochondria when expressed at high levels through either overexpression or inhibition of MCL-1 degradation using proteasome inhibitors (10, 13), similar to our observation that high levels of MCL-1 could inhibit BAX conformational change. On the other hand, levels of MCL-1 closer to the endogenous amount found in HeLa cells (MCL-1 (low)) did not prevent this initial activation step or BAX translocation to mitochondria. However, low levels of MCL-1 were sufficient to inhibit the formation of the high molecular mass BAX oligomers (>200 kDa) that have been associated with cytochrome c release (20), consistent with the ability of MCL-1 to inhibit BAX-dependent cytochrome c release. Of note, the apoptotic resistance of these cells could be reverted using Ad Noxa (Fig. 5B) or siRNA against MCL-1 (data not shown), indicating that it is dependent on MCL-1 expression rather than on other non-specific effects arising from the selection of the stable cells.

The inhibition of BAX-induced cytochrome c release by MCL-1 in the absence of interactions between the two proteins could occur through several possible mechanisms. One explanation would be that MCL-1 acts through BAK because the two proteins readily interact. However, this is unlikely, as the downregulation of BAX did not prevent MCL-1-dependent inhibition of BAX. In addition, the activation of endogenous BAX by transfected BAX is unlikely to be physiologically relevant, as down-regulation of BAX did not affect the activation of endogenous BAX in UV light-treated HeLa cells (data not shown). Rather, it is likely to be the result of the fact that, whereas BAX is mitochondrial, BAX is cytosolic and, upon its translocation to mitochondria following overexpression, could activate both itself and BAK (3).

A second possibility stems from the fact that in the MCL-1 (low) cells BAX formed small complexes that did not contain MCL-1. These could represent partially oligomerized BAX that is prevented from further oligomerization because MCL-1 inhibits a BAX activator. However, MCL-1 did not influence Bif1 or DRP1, two proteins that are involved in BAX-dependent cytochrome c release. One other interpretation is that the partial complexes contain BAX associated with an inhibitor, consistent with the idea that, once activated, BAX needs to be kept in check to prevent its oligomerization and subsequent formation of cytochrome c release channels (3, 18). Interestingly, BAX complexes cofractionated with BCL-XL-containing complexes, suggesting that activated BAX might be sequestered by BCL-XL in these cells. This would be consistent with the reported increase in affinity between BAX and anti-apoptotic BCL-2 homologues when BAX is activated (3, 11). This is also supported by the observation that BAD was able to reverse the effect of MCL-1 (low) on cytochrome c release. The indirect effect of MCL-1 on other BCL-2 homologues observed here also provides an explanation for the fact that MCL-1 degradation is required for apoptosis to occur following UV irradiation (13), MCL-1 promoting in this case the inactivation of BAX by a BAD-inhibited BCL-2 homologue. BH3-only proteins would be possible MCL-1 targets in this context. Decreasing levels of MCL-1 would release increasing amounts of activator BH3-only proteins to activate BAX. On the other hand, overexpression of MCL-1, even at low levels, would not leave enough free BH3-only proteins to inactivate BCL-XL (or BCL-2/BCL-W), which would then be free to inhibit the active conformer of BAX, similar to the inhibition of BAK by BCL-2 (18).

Finally, several experimental approaches were used to address the contribution of BAX to the function of MCL-1. Results obtained using MCL-1 (−/−) MEFs suggested that MCL-1 regulates BAK to some extent, in accordance with the direct interaction between the two proteins. However, the effects on BAX were much greater and were observed both in the absence of MCL-1 and following its overexpression. This suggests that the presence of BAX is important for the anti-apoptotic activity of MCL-1 and that BAX is a major downstream target of MCL-1. Independent of the actual mechanism through which MCL-1 inhibits BAX-dependent cytochrome c release, this is likely to be of functional importance for the pro-survival effect of MCL-1.

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