MAP-1 is a mitochondrial effector of Bax

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Edited by Xiaodong Wang, University of Texas Southwestern Medical Center, Dallas, TX, and approved August 28, 2005 (received for review May 5, 2005)

Apoptotic stimuli induce conformational changes in Bax and trigger its translocation from cytosol to mitochondria. Upon assembling into the mitochondrial membrane, Bax initiates a death program through a series of events, culminating in the release of apoptogenic factors such as cytochrome *c*. Although it is known that Bax is one of the key factors for integrating multiple death signals, the mechanism by which Bax functions in mitochondria remains controversial. We have previously identified modulator of apoptosis-1 (MAP-1) as a Bax-associating protein, but its functional relationship with Bax in contributing to apoptosis regulation remains to be established. In this study, we show that MAP-1 is a critical mitochondrial effector of Bax. MAP-1 is a mitochondria-enriched protein that associates with Bax only upon apoptotic induction, which coincides with the release of cytochrome *c* from mitochondria. Small interfering RNAs that diminish MAP-1 levels in mammalian cell lines confer selective inhibition of Bax-mediated apoptosis. Mammalian cells with stable expression of MAP-1 small interfering RNAs are resistant to multiple apoptotic stimuli in triggering apoptotic death as well as in inducing conformation change and translocation of Bax. Similar to Bax-deficient cells, MAP-1-deficient cells exhibit aggressive anchorage-independent growth. Remarkably, recombinant Bax- or tBid-mediated release of cytochrome *c* from isolated mitochondria is significantly compromised in the MAP-1 knockdown cells. We propose that MAP-1 is a direct mitochondrial target of Bax.

apoptosis | mitochondria | Bcl-2 family | tumor suppressor

Proteins of the Bcl-2 family are central regulators of survival and apoptotic signals through mitochondria by affecting permeabilization of mitochondria outer membrane, and thereby regulating the release of death-promoting factors (1–4). The Bcl-2 family consists of three subfamilies of prosurvival or proapoptotic molecules. Most members of the BH3-only subfamily of proapoptotic molecules act by relaying distinct death signals to the mitochondria through binding to members of the multidomain prosurvival subfamily (e.g., Bcl-2 and Bcl-X_L), whereas the BH3-only molecule, Bid, appears to have an additional role in promoting activation of members of the multidomain proapoptotic subfamily such as Bax and Bak (3–5).

Murine embryonic fibroblasts (MEFs) with *Bax* or *Bak* deleted displayed no defect in apoptosis (4, 6). However, MEFs with double *Bax* and *Bak* knockouts showed dramatic resistance to diverse apoptotic stimuli, suggesting that Bax and Bak are central, but redundant, regulators of apoptosis signaling (6, 7). However, analyses of apoptosis signaling events in neurons obtained from knockout animals and human cell lines and tumors suggest that Bax might exert a dominant function over Bak in certain cell contexts (8–12).

Bak is a resident protein in mitochondria, and its proapoptotic activity is restrained by associating with VDAC2 (13) or Mcl-1 (14, 15). In contrast to Bak, Bax is predominantly localized in cytosol or loosely attached to mitochondrial membranes in an inactive form in healthy cells (16). Apoptotic stimuli cause the unfolding of N and C termini of Bax (17–19). These structural changes may facilitate translocation of Bax from cytosol to mitochondria where Bax oligomerizes into a high molecular weight complex, leading to permeabilization of the mitochondrial outer membranes (20–22). Interestingly, other studies suggest that conformation changes and translocation of Bax to mitochondria alone are insufficient for engaging its molecular function in mitochondria (23, 24).

It has recently been shown that tBid, Bax, and a defined lipid environment were sufficient to reconstitute some properties of mitochondrial membrane permeabilization (MMP) (22). However, this artificial system does not recapitulate all of the properties of MMP (22, 25). Indeed, additional unidentified factor in mitochondria is needed for Bax function in mitochondria (24).

Because Bax was shown to interact with VDAC (26) and adenine nucleotide translocator (27), which constitute, in part, the mitochondrial permeability transition pore (MPTP), it was proposed that Bax may function in mitochondria by facilitating the opening of MPTP, and the opening of MPTP may eventually lead to mitochondrial swelling, rupture of the outer membrane, and subsequent release of cytochrome *c* (Cyto *c*). However, recent work in characterizing the phenotypes of *cyclophilin D* knockout animals casts doubt on the idea (28–30). *Cyclophilin* $D^{-/-}$ cells are severely defective in MPTP opening stimulated by necrotic, but not apoptotic stimuli (28, 29). Moreover, mitochondria isolated from cyclophilin $D^{-/-}$ cells exhibited no defect in the effect of recombinant Bax- or tBid-induced Cyto *c* release (28, 29). Here, we present evidence to demonstrate that modulator of apoptosis-1 (MAP-1) is a critical effector for Bax function in mitochondria.

Materials and Methods

Immunoblotting, Immunoprecipitation, and Indirect Immunofluorescence. Western blotting and immunoprecipitation were performed as described (31). For coimmunoprecipitation of endogenous MAP-1 and Bax, MCF-7 cells were resuspended in lysis buffer (20 mM Tris·HCl, pH 7.5/150 mM NaCl/2 mM EDTA/10% glycerol/2% CHAPS) and homogenized. Immunoprecipitation and confocal analysis of active Bax was performed by using anti-Bax conformation-dependent antibody (N20, Santa Cruz Biotechnology) as described (23, 32, 33). The rabbit (R5) and mouse (M6) anti-MAP-1 polyclonal antibodies were raised against bacterial GST-MAP-1 (amino acids 116–351) protein. Indirect immuofluorescence was performed according to the procedure described by Chua *et al.* (34).

Subcellular Fractionation. Mitochondria, endoplasmic reticulum, and cytosolic fraction were isolated as described earlier (34). Nuclei were isolated as described (31). Where indicated, mitochondria-enriched fraction was further purified through sucrose gradients essentially as described (21, 35).

Generation of Stable MAP-1 RNA Interference (RNAi) Knockdown and Rescue Lines. MCF-7 and HCT116 cells were transfected with p*Silencer* Hygromycin or p*Silencer* G418 MAP-1 short hairpin RNA (shRNA) constructs and selected with 400 μg/ml hygromycin B (Invitrogen) or 1.25 mg/ml G418 (GIBCO), respectively. Individual clonal lines were evaluated for the knockdown efficiency of

This paper was submitted directly (Track II) to the PNAS office.

Freely available online through the PNAS open access option.

Abbreviations: MAP-1, modulator of apoptosis-1; Cyto *c*, cytochrome *c*; prot K, proteinase K; siRNA, small interfering RNA; STS, staurosporine.

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MAP-1 protein and used for further analysis. See *Supporting Text*, which is published as supporting information on the PNAS web site, for details of the construction of small interfering RNA (siRNA) expression plasmids and their target sequences in human MAP-1 mRNA. To generate rescue lines in MAP-1 RNAi knockdown background, the MCF-7 MAP-1 knockdown clonal line SM-R3-12 was transfected with pIRESneo vector or pIRESneo MAP-1 construct with three silent mutations within the region targeted by shRNA R3 (TTACTGTTGACGAATGCCT) and selected in 300 μg/ml hygromycin B (Invitrogen) plus 1.25 mg/ml G418 (GIBCO). The individual clonal lines were evaluated for the expression of myc-MAP-1.

Apoptosis Assays. To detect apoptosis in transfected cells, Bax, Bak, or MAP-1 construct was cotransfected with GFP reporter, and GFP-positive apoptotic nuclei were scored as described (34). To measure apoptosis in stable lines, viability was determined by JC-1 (Molecular Probes), WST-1 (Roche Diagnostics), or caspase activity (Calbiochem) according to the respective manufacturer's instructions. The clonogenicity assay was performed essentially as described (34).

***In Vitro* Cyto *c* Release.** Equal amounts of mitochondria were incubated with recombinant *m*-tBid (amino acids 60–195), hBax (full length), or hBak delta TM (amino acids 1–190) proteins at 30°C for 30 min followed by centrifugation. Supernatants and pellets were subjected to Western blotting analysis. All recombinant proteins were generated under detergent-free conditions as described (17). See *Supporting Text* for details of the preparation of recombinant proteins.

Results

MAP-1 Is a Mitochondria-Enriched Protein. MAP-1 was initially identified in a yeast two-hybrid screen using Bax as bait (31). To determine the subcellular localization of endogenous MAP-1, 293T cells were fractionated into cytosol, nuclei, light membrane, and heavy membrane (HM) fractions and immunoblotted with MAP-1 and other antibodies as indicated. The 39-kDa MAP-1 protein was detected predominantly in the HM fraction enriched with the mitochondria marker (Fig. 1*A Left*). Moreover, MAP-1 and Cyto *c* were also readily detected in the sucrose gradient-purified mitochondria (Fig. 1*A Right*).

A series of deletion mutants of MAP-1 were made to determine the sequence requirement for mitochondrial targeting. The wild-type MAP-1 and the MAP-1(1–115) mutant were found to be highly enriched in the HM fraction, whereas all of the N-terminal deletion mutants were cytosolic, suggesting that the N-terminal region of MAP-1 is necessary for targeting MAP-1 to mitochondria (Fig. 1*B*). Further fine mapping of the mitochondrial targeting sequence was hindered because some deletion mutants were poorly expressed.

Although both R5 and M6 antibodies were able to detect overexpressed myc-MAP-1 with similar efficiency as myc antibody in mammalian cells, neither antibody was able to detect a specific immunofluorescence signal corresponding to endogenous MAP-1 in MCF-7, 293T, or SH-SY5Y cells, suggesting that MAP-1 protein is at low abundance. To further confirm the mitochondrial localization of MAP-1, we used confocal microscopy to visualize transiently expressed myc-MAP-1 in MCF-7 cells. Myc-MAP-1 is proapoptotic and exhibited a perinuclear staining pattern that colocalized with the mitochondria-specific dye, mitotracker (Fig. 1*C Upper*). In contrast, the nonapoptotic form of MAP-1, MAP-1(1–115) (31), displayed excellent colocalization with mitotracker (Fig. 1*C Lower*). To investigate further the association of MAP-1 with mitochondria, *in vitro* translated ^{35}S-labeled proteins were incubated with isolated mitochondria. MAP-1 and Bcl-X_L, but not the cytosolic protein hFEM-2, were found to readily associate with isolated mitochondria (Fig. 1*D*).

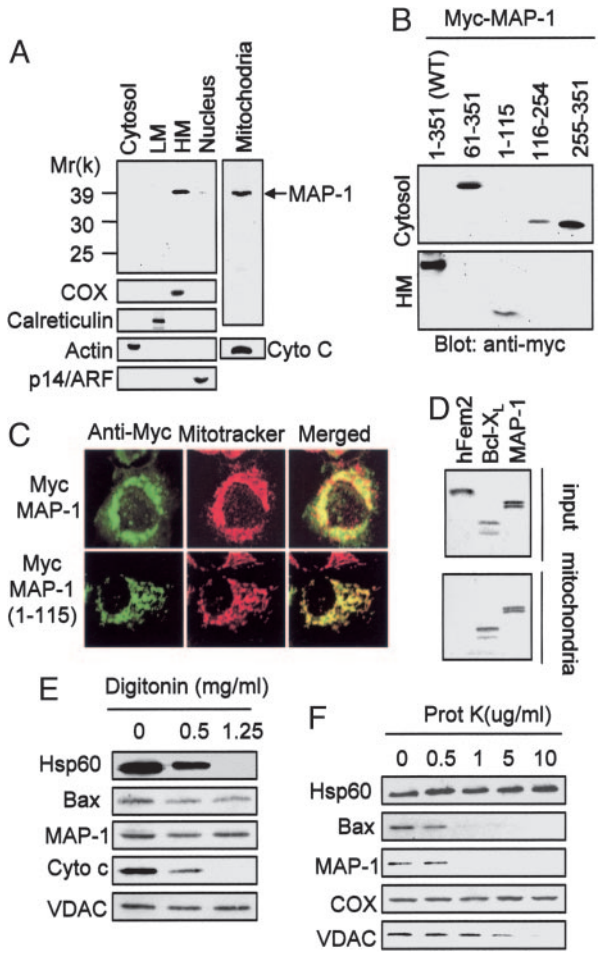

Fig. 1. MAP-1 is a mitochondrial protein residing at outer membrane. (*A*) (*Left*) Subcellular fractions from 293T cells were immunoblotted with the MAP-1 antibody (R5) or antibodies against the organelle-specific protein markers: COX (mitochondria), calreticulin (ER), actin (cytosol), and p14/ARF (nuclear). (*Right*) Sucrose gradient purified mitochondria from 293T cells were immunoblotted with R5 or anti-Cyto *c* antibody. (*B*) The N-terminal region of MAP-1 is required for mitochondrial targeting. The Myc-MAP-1 deletion mutants were transiently transfected into MCF-7 cells. Lysates from transfected cells were subjected to fractionation analysis as in *A*. HM and cytosolic fractions were immunoblotted with anti-myc antibody. (*C*) Myc-MAP-1 and the MAP-1 mutant (1–115) colocalize with mitotracker. MCF-7 cells were transiently transfected with Myc-MAP-1 or Myc-MAP-1 mutant (1–115). Sixteen hours after transfection, cells were stained with mitotracker (red) and anti-myc (green). (*D*) MAP-1 associates with isolated mitochondria. The indicated *in vitro* translated ^{35}S-labeled proteins were incubated with mitochondria isolated from MCF-7 cells at 25°C for 20 min. Mitochondria were washed twice and repelleted by centrifugation. (*E*) MAP-1 is a membrane bound mitochondrial protein. Mitochondria were incubated with indicated concentration of digitonin on ice for 30 min. Mitochondria were repelleted by centrifugation (10,000 $\times$ *g*; 10 min) and immunoblotted for the indicated protein. (*F*) Mitochondrial MAP-1 is highly sensitive to prot K digestion. Mitochondria were incubated with indicated concentration of prot K on ice for 10 min. PMSF (10 mM) was added to stop prot K digestion. Mitochondria were repelleted by centrifugation and immunoblotted for the indicated protein.

To study the submitochondrial localization of MAP-1, mitochondria were subjected to digitonin and proteinase K (prot K) treatments. Although mitochondrial matrix protein Hsp60 and intermembrane space protein Cyto *c* were readily released into supernatant, the level of MAP-1 remained unchanged upon digitonin treatment, suggesting that MAP-1, similar to Bax and VDAC, is a membrane-associating protein (Fig. 1*E*). In contrast to Hsp60

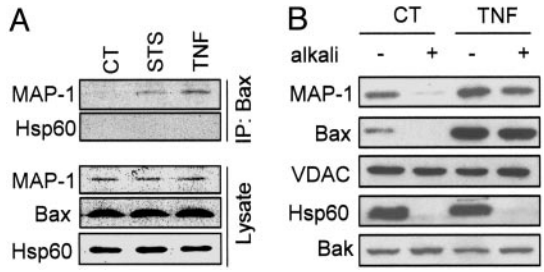

Fig. 2. Apoptotic stimuli induce association of Bax with MAP-1 and promote their integration to mitochondrial membrane. (*A*) Apoptotic stimuli promote endogenous Bax-MAP-1 association. MCF-7 cells were treated with STS (1 μM, 5 h) or TNF (40 ng/ml, 7 h). Cell lysates were immunoprecipitated with anti-Bax antibody. Immunoprecipitates were immunoblotted with MAP-1 (M6) or Hsp60 antibody (*Upper*). Total lysates were immunoblotted with the indicated antibodies (*Lower*). (*B*) Apoptotic stimuli promote integration of both MAP-1 and Bax into mitochondrial outer membrane. Mitochondria isolated from control or TNF-treated (40 ng/ml, 7 h) MCF-7 cells were resuspended in 0.1M Na_2CU_3 (pH 11.5), where indicated, and incubated on ice for 20 min followed by sonication for 5 min. Mitochondria were repelleted by centrifugation (350,000 $\times$ *g*, 20 min) and immunoblotted for the indicated protein.

and the inner membrane integrated protein COX 4, MAP-1, Bax, and the outer membrane integrated protein VDAC were all sensitive to prot K digestion, suggesting that MAP-1 associates mainly with mitochondrial outer membrane (Fig. 1*F*).

MAP-1 Interacts with Bax During Apoptosis. Apoptotic stimuli are known to promote translocation of Bax to mitochondria. We examined the possibility that Bax and MAP-1 may colocalize and associate only during apoptosis. Upon apoptosis induction, excellent colocalization patterns for MAP-1 and Bax were detected (Fig. 7*A*, which is published as supporting information on the PNAS web site). Although MAP-1 was previously shown to be a Bax-associating protein, endogenous Bax/MAP-1 association has not been demonstrated (31). Endogenous MAP-1 was found to coimmunoprecipitate with endogenous Bax only in staurosporine (STS)- or TNF-treated cells, but not in healthy cells (Fig. 2*A*). The time course of Bax/MAP-1 association coincided with the appearance of Cyto *c* in cytosol during apoptosis (Fig. 7*B*). Interestingly, although mitochondrial MAP-1 and Bax from healthy cells were completely removed by alkali extraction, mitochondrial MAP-1 and Bax from TNF-treated (Fig. 2*B*) or STS-treated (data not shown) cells became resistant to alkali, suggesting that apoptotic stimuli promote the integration of both Bax and MAP-1 into the mitochondrial outer membrane.

MAP-1 Is Required for Bax-Induced Apoptosis Signaling. The observation that Bax and Bak serve a completely redundant function in apoptosis signaling in murine embryonic fibroblasts (7) raises the possibility that they may signal through a similar mechanism in mitochondria. Interestingly, although MAP-1 is a binding partner for Bax during apoptosis, association of MAP-1 with Bak was not observed even under overexpression condition (data not shown). To permit evaluation of the possible role for MAP-1 in Bax- and Bak-mediated apoptosis signaling, we used siRNA to silence the expression of MAP-1. MAP-1 siRNAs R1 or R3, but not scrambled siRNA (Scr), significantly reduced endogenous MAP-1 protein (>80%) (Fig. 8*A*, which is published as supporting information on the PNAS web site). Silencing MAP-1 by transient transfection of R1 or R3 siRNA in MCF-7 cells inhibited Bax-mediated cell death, as determined by counting the percentage of cells with nuclear condensation in GFP-positive cells (Fig. 3*A*) or by a more objective assay measuring the luciferase reporter activity to monitor the survival of transfected cells (Fig. 8*B*). In contrast, diminishing MAP-1 levels in these cells failed to inhibit Bak-induced cell death

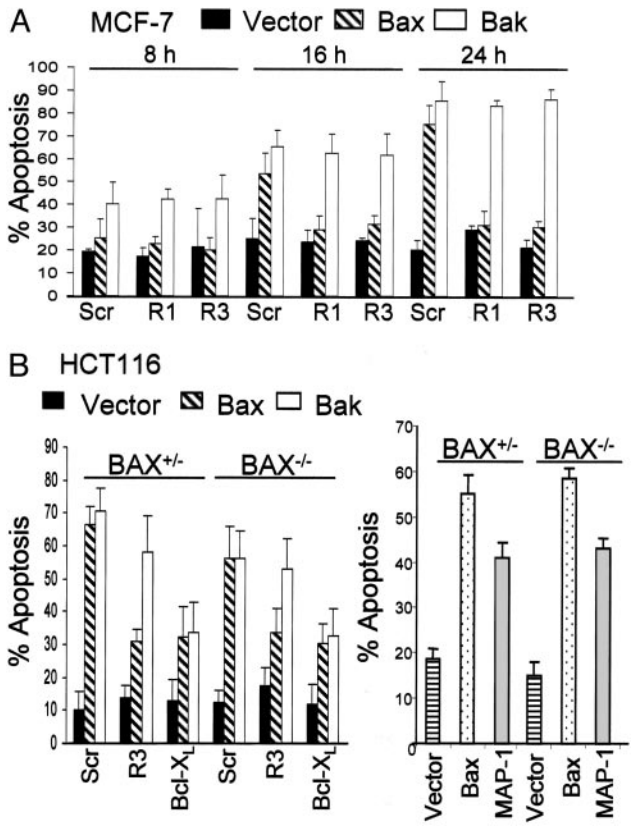

Fig. 3. MAP-1 is required for Bax-induced, but not Bak-induced, apoptosis. (*A*) MCF-7 cells were first transfected with scramble siRNA (Scr) or MAP-1 siRNAs R1 or R3 for 2 days followed by a second transfection with pXJ40 vector, HA-Bax, or HA-Bak together with the GFP reporter. Apoptosis was determined by counting the percentage of GFP-positive cells that exhibited condensed nuclei morphology. (*B*) (*Left*) HCT116 $Bax^{+/-}$ or $Bax^{-/-}$ cells were subjected to identical transfection protocols as in *A*, and the cells were processed 18 h after the second transfection. (*Right*) MAP-1 induces apoptosis in $Bax^{+/-}$ and $Bax^{-/-}$ cells with similar efficacy. HCT116 $Bax^{+/-}$ or $Bax^{-/-}$ cells were transiently transfected with either pXJ40 vector, HA-Bax, or Myc-MAP-1. The percentage apoptosis was determined as in *A*. Results are presented as percentage of control (mean $\pm$ SD, $n = 3$).

(Fig. 3*A*). Further experiments performed in 293T and SH-SY5Y cells with the R1 or R3 siRNAs gave similar results (data not shown).

To further determine the functional relationship between MAP-1 and Bax, we performed experiments in isogenic HCT116 cell lines with differing Bax genotypes. The R3 siRNA was effective in lowering MAP-1 levels in both $Bax^{+/-}$ and $Bax^{-/-}$ lines (Fig. 8*A*). Similar to MCF-7 cells, silencing MAP-1 in these cells conferred selective resistance to Bax-induced apoptosis (Fig. 3*B Left*). Interestingly, MAP-1 overexpression triggered apoptosis to similar degree in both $Bax^{+/-}$ and $Bax^{-/-}$ lines (Figs. 3*B Right* and 8*C*), suggesting that MAP-1 may act as a downstream effector of Bax and can be spontaneously activated when it reaches relatively high level in the cells.

Silencing MAP-1 in MCF-7 Cells Confers Resistance to Diverse Apoptotic Stimuli. To expand our analyses of the role of MAP-1 in apoptosis signaling, we generated stable MCF-7 lines harboring either R1 or R3 siRNA. As parallel controls, we generated stable lines carrying siRNAs with two nucleotide mutations from the siRNA sequences (R1 mut and R3 mut). The MAP-1 levels in the SM-R3-12 clonal line was dramatically reduced (>80%), whereas the levels of Bax appeared unchanged (Fig. 4*A*). MCF-7 cells

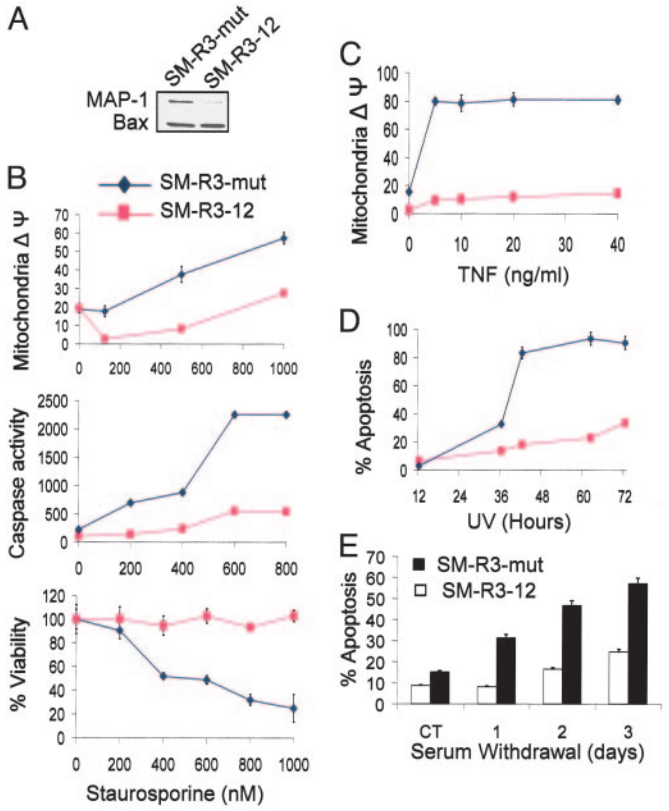

Fig. 4. MAP-1 knockdown MCF-7 cells are resistant to diverse apoptotic stimuli. (*A*) MAP-1 protein level is substantially reduced in SM-R3-12 cells stably expressing the MAP-1 siRNA. Cell lysates were immunoprecipitated with Bax (N20) or MAP-1 (R5) antibodies followed by immunoblotting with MAP-1 (M6) or Bax (2D2) antibodies. (*B*) MAP-1 knockdown cells are resistant to apoptotic death triggered by STS. (*Top*) Cells were harvested and stained with JC-1 for analysis of mitochondrial membrane potential change by flow cytometry. (*Middle*) The extent of caspase activation following treatment with STS was assayed with AC-DEVD-AFC. (*Bottom*) Cell viability after STS treatment was determined by WST-1 assay. (*C–E*) MAP-1 knockdown cells displayed resistance to apoptosis triggered by TNF (*C*), UV (*D*), and serum withdrawal (*E*). Cells were subjected to various apoptotic insults as indicated and the dose-dependent cell viability responses were determined by the following assays: JC-1 (TNF) or trypan blue exclusion (UV and serum withdrawal). Results are presented as percentage of control (mean ± SD, $n = 3$).

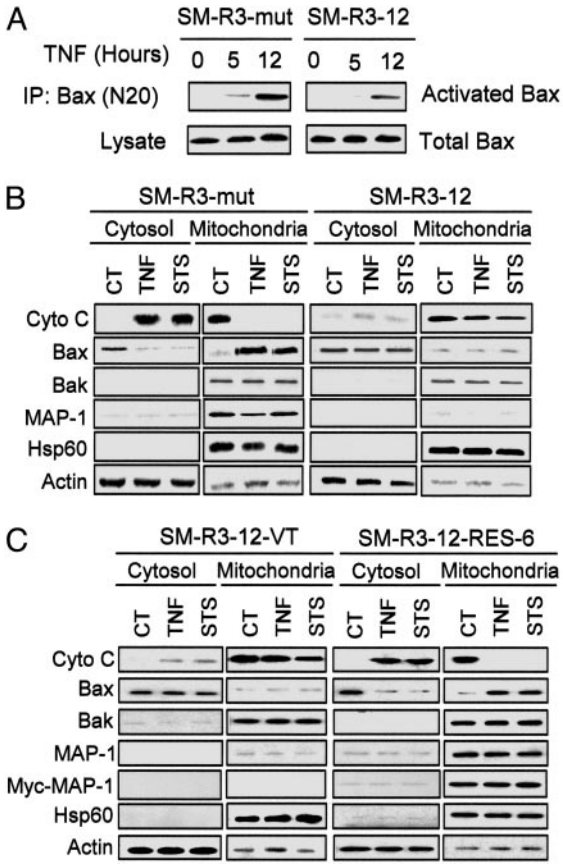

Fig. 5. Apoptotic stimuli-mediated conformation change and translocation of Bax are inhibited in MAP-1-depleted cells. (*A*) TNF-mediated conformation change of Bax was affected in MAP-1 knockdown cells. Equal amounts of total lysates from the TNF-treated (40 ng/ml) cells were immunoprecipitated with the conformation-specific anti-Bax antibody, N20. Cell lysates and immunoprecipitates were immunoblotted with anti Bax. (*B*) MAP-1 is required for apoptosis-mediated Bax translocation and Cyto *c* release. Cytosolic and mitochondrial fractions from cells treated with either STS (1 μM, 5 h) or TNF (40 ng/ml,10 h) were immunoblotted with the indicated antibodies. (*C*) Stable expression of myc-MAP-1 in the MAP-1 knockdown cells is sufficient for restoring the sensitivity of MAP-1-deficient cells to apoptotic stimuli-mediated Bax translocation and Cyto *c* release. Cells were treated and analyzed as in *B*.

lacking MAP-1 were healthy and displayed normal morphology (data not shown). Upon STS treatment, SM-R3-mut cells displayed typical apoptotic changes, such as diffuse Cyto *c* staining (data not shown), nuclear condensation (data not shown), a rapid drop in mitochondrial membrane potential (Fig. 4*B Top*), and caspase activation (Fig. 4*B Middle*), and rapidly lost viability as demonstrated by WST-1 assay (Fig. 4*B Bottom*). In contrast, SM-R3-12 cells were resistant to STS-mediated changes associated with cell death (Fig. 4*B*) and, even when exposed to STS for 72 h, a substantial portion of the cells remained viable (data not shown). Remarkably, the MAP-1 knockdown cells were resistant to diverse apoptotic stimuli including TNF (Fig. 4*C*), UV irradiation (Fig. 4*D*), serum withdrawal (Fig. 4*E*), and TNF-related apoptosis-related ligand (TRAIL) (data not shown) in a variety of assays (Fig. 4 and data not shown). The MAP-1 knockdown MCF-7 cells that survived the STS or TNF treatment displayed long-term survival and were subsequently able to form 60–70% more colonies in a clonogenecity assay than the control cells that were similarly treated (data not shown). The specificity of the effects was further studied by analyzing two additional independent stable lines expressing the R3 MAP-1 siRNA and three other independent stable clonal lines expressing a completely different MAP-1 siRNA, R1, for their sensitivity toward STS and TRAIL-induced killing; similar results were obtained (Fig. 9*C*, which is published as supporting information on the PNAS web site, and data not shown). MAP-1-deficient HCT116 cells are significantly more resistant than control cells to apoptotic effects triggered by STS or TRAIL (Fig. 10*B*, which is published as supporting information on the PNAS web site), suggesting that the effects associated with knocking down MAP-1 observed in MCF-7 cells can be extended to other cell types.

Conformation Change and Translocation of Bax Triggered by Apoptotic Stimuli Are Inhibited in MAP-1-Deficient Cells. Next, we examined whether MAP-1 is required for mediating conformation change and translocation of Bax during apoptosis. Upon TNF treatment, the conformation-specific Bax antibody revealed a clear difference in the kinetics of the effect on conformational change in Bax between the SM-R3-mut and the SM-R3-12 cells. The change was detected 5 h after TNF treatment in SM-R3-mut cells, whereas it was only detectable in SM-R3-12 cells after 12 h of treatment (Fig. 5*A*). As shown in fractionation analysis, TNF or STS effectively induced translocation of Bax from the cytosol to mitochondria as

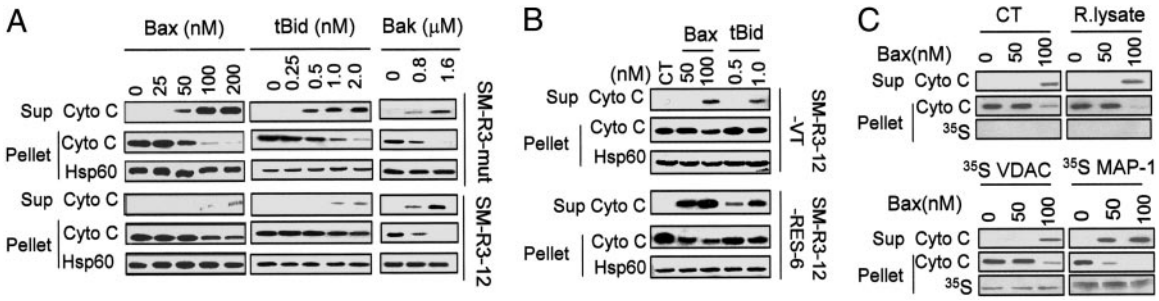

Fig. 6. MAP-1 is required to facilitate Bax- and tBid-mediated release of Cyto *c* from isolated mitochondria. (*A*) Mitochondria isolated from control or MAP-1 knockdown cells were incubated with recombinant Bax, tBid, or Bak followed by centrifugation. The supernatants and pellets were immunoblotted with indicated antibodies. (*B*) Stable expression of myc-MAP-1 in MAP-1 knockdown cells restores the sensitivity of mitochondria to Bax- and tBid-induced release of Cyto *c*. Mitochondria isolated from MAP-1 knockdown or rescue cells were treated and analyzed as in *A*. (*C*) *In vitro* translated MAP-1 restores the sensitivity of MAP-1-deficient mitochondria to Bax-mediated release of Cyto *c*. Mitochondria from MAP-1 knockdown cells were preincubated with rabbit reticulate lysate (R. lysates), ^{35}S-labeled *in vitro* translated MAP-1, or VDAC (1×10^5 cpm) for 20 min at 25°C. The mitochondria was washed twice, treated with recombinant Bax, and analyzed as in *A*.

well as Cyto *c* from the mitochondria to the cytosol in SM-R3-mut cells (Fig. 5*B*). In contrast, Bax remained largely cytosolic upon apoptotic treatments in SM-R3-12 cells (Fig. 5*B*). The conformation change and translocation associated with Bax activation were further studied by using confocal microscopy. TNF-treated, GFP-Bax-positive cells from the SM-R3-mut line stained by the N20 antibody were more readily seen (>80%) than those from the SM-R3-12 line (<30%) (Fig. 11*A*, which is published as supporting information on the PNAS web site). Upon treatment with TNF, the initial diffuse staining of cytosolic GFP-Bax in control cells, but not MAP-1 knockdown cells, readily assumed a punctuate staining pattern consistent with the release of Cyto *c* from mitochondria (Fig. 11*B*, which is published as supporting information on the PNAS web site). TNF-induced Bax translocation occurred in >80% of control cells but in <30% of MAP-1 knockdown cells (Fig. 11*B*).

The phenotype associated with MAP-1-deficient cells is indeed a direct consequence of a reduction in MAP-1 protein level, because stable clonal rescue lines expressing myc-MAP-1 (SM-R3-12-RES) in the background of SM-R3-12 cells were found to regain sensitivity toward STS- and TNF-related apoptosis-related ligand-mediated killing (Fig. 12, which is published as supporting information on the PNAS web site). Furthermore, the translocation defect of Bax noted in MAP-1 knockdown cells (Fig. 5*B*) was no longer detectable in the clonal rescue lines (Fig. 5*C* and data not shown).

MAP-1 Has a Direct Role in Facilitating Bax Function in Releasing Apoptogenic Factors from Mitochondria. Recombinant tBid, Bax, and Bak are capable of releasing Cyto *c* directly from isolated mitochondria (36–38). To investigate whether MAP-1 has a direct role in mediating Bax function in mitochondria, we evaluated and compared the effects of recombinant Bax, Bak, and tBid proteins in directly releasing Cyto *c* from isolated mitochondria derived from the control and MAP-1 knockdown cells. Purified recombinant Bax, Bak, and tBid proteins were all able to release Cyto *c* from isolated mitochondria prepared from SM-R3-mut and SH-R3-mut cells (Figs. 6*A* and 10 *C* and *D*). Interestingly, the activity of both Bax (Figs. 6*A* and 10*C*) and tBid (Fig. 6*A* and 10*D*) in releasing Cyto *c* from isolated mitochondria were all severely compromised in MAP-1 knockdown cells. In contrast, no significant difference was noted in the Cyto *c* releasing activity of recombinant Bak on mitochondria isolated from MAP-1 knockdown cells (Fig. 6*A* and data not shown). The ability of Bax and tBid, but not Bak, in releasing Smac/DIABLO (1–3) from mitochondria was similarly affected in MAP-1 knockdown cells (data not shown). Further analyses of two additional stable clonal lines derived from R3 and three from R1 siRNA with respect to the Cyto *c*-releasing function of recombinant Bax protein yielded similar results (Fig. 9*B* and data not shown).

The dampened sensitivity of MAP-1-deficient mitochondria to Bax- or tBid-mediated release of Cyto *c* was largely restored in the stable myc-MAP-1 rescue lines (Fig. 6*B* and data not shown). Furthermore, incubation of MAP-1-deficient mitochondria with *in vitro* translated MAP-1, but not reticulate lysate or *in vitro* translated VDAC, was able to restore the sensitivity of MAP-1-deficient mitochondria to Bax-mediated Cyto *c* release (Fig. 6*C*).

Discussion

The structural data of Bax revealed that the putative transmembrane domain (helix α9) masks the hydrophobic cleft, which has structural features similar to the BH3 ligand-binding groove formed by the BH1–3 domains of Bcl-X_L (17, 39, 40). Apoptotic stimuli trigger disengagement of helix α 9 from the hydrophobic pocket. Interestingly, the only motif identifiable in MAP-1 is a BH3-like domain (31). A single point mutation of the conserved amino acid in any one of the three BH domains of Bax was shown to be sufficient for abolishing its binding to MAP-1, suggesting that the hydrophobic cleft of Bax could be the binding pocket for MAP-1 (31).

Endogenous MAP-1 only interacts with Bax during apoptosis, and the MAP-1 knockdown has no significant effect on Bak-mediated apoptosis in transient transfection experiments. Interestingly, despite the presence of Bak in the MCF-7 and HCT116 cells, abolishing MAP-1 in these cells was sufficient to confer significant resistance to multiple apoptotic stimuli. Thus, our data support the idea concluded from other studies that Bax may not be serving completely redundant function to Bak and that it may have a dominant function over Bak in regulating the central apoptosis signaling in certain cellular contexts (8–12).

Similar to Bak, mitochondrial MAP-1 does not appear to mobilize to cytosol during apoptosis. Thus, it seems surprising that MAP-1 could affect the conformation change and translocation of Bax. However, Bcl-X_L and Bcl-2, which localize and act primarily in the mitochondria, are also known to be effective in inhibiting conformation change and translocation of Bax triggered by apoptotic stimuli (32, 33). Therefore, it is possible that a signal amplification cascade is at work in creating a positive feedback loop in driving a continuous and sustained activation of the mitochondrial signaling pathway during apoptosis.

The *in vitro* Cyto *c* release data from MAP-1-deficient mitochondria strongly suggest that MAP-1 is a critical factor for the Cyto *c* releasing function of Bax in mitochondria. It is surprising to note that MAP-1, which appears to be necessary only for Bax-mediated, but not Bak-mediated, apoptosis signaling, is also required for tBid

to release Cyto *c* from isolated mitochondria, because it is thought that tBid can engage mitochondrial apoptosis signaling by direct activation of either Bax or Bak and facilitating formation of homo-oligomers (20, 41). In cells where Bax has a dominant role over Bak, an intact Bax-MAP-1 pathway may actually be necessary for efficient activation of Bak by tBid. Indeed, a recent study demonstrated that effective oligomerization of Bak depends on Bax, rather than tBid (42). The ability of tBid, but not Bak, in releasing Cyto *c* from isolated mitochondria was also found to be severely inhibited in HCT116 $Bax^{-/-}$ cells (data not shown; ref. 43), which is one of the cell types known to display a dominant Bax function (9–11, 43). Moreover, recent data revealed that tBid appears to be a potent displacer of Bax from Bcl-X_L, but not a displacer of Bak from Mcl-1 (44). These data are therefore in line with the observations that releasing of Cyto *c* from isolated mitochondria by tBid could be severely inhibited in the absence of either MAP-1 or Bax.

Bax is frequently inactivated in tumors of the microsatellite mutator phenotype, which comprise 15% of human colon, gastric, and endometrial cancers (45, 46). Inactivation of the Bax gene confers an obvious selective advantage for tumor growth during clonal evolution (9, 45). If MAP-1 is indeed a crucial target for Bax-mediated signaling, MAP-1 knockdown cells should acquire similar growth advantages exhibited by $Bax^{-/-}$ cells (46). Indeed, MAP-1 knockdown cells formed foci aggressively on soft agar (Fig. 10*E* and Fig. 13*A*, which is published as supporting information on the PNAS web site), suggesting that MAP-1 may have a role in suppressing anchorage-independent growth in tumor cells. Furthermore, MAP-1-deficient MCF-7 cells xenografted to athymic nude mice resulted in significantly larger tumors than those derived from the control cells (Fig. 13*B*).

While our manuscript was being reviewed, Baksh *et al.* (47) published their interesting finding that the tumor suppressor, RASSF1A, can specifically link death receptor signaling to Bax activation through binding to MAP-1. Their finding provides a putative mechanism to account for the role of MAP-1 in mediating Bax conformation change in certain cell types in response to the death receptor signals. Interestingly, a recent report suggests that endogenous protein products of *RASSF1* (1A, 1B, and 1C) are predominantly localized in mitochondria (48). Baksh *et al.* (47) demonstrated that loss of RASSF1A in mammalian cells resulted in dampening of apoptotic effect triggered by the death receptor signaling. Our study showed that knocking down MAP-1 in mammalian cells confers inhibition of apoptosis signaling triggered by multiple stimuli and promotes anchorage independent and tumor growth. Moreover, molecular analysis of MAP-1 function suggests that MAP-1 has a significant role as an effector of Bax in mitochondria. The current study, together with the finding of Baksh *et al.* (47), underscore the important role of MAP-1 in apoptosis signaling and lend further support to the idea that MAP-1 could be a key regulator in a tumor suppressor axis.

We are grateful to Dr. Bert Vogelstein (Johns Hopkins University, Baltimore) for providing us with the HCT116 cell lines. We thank Drs. Bor Luen Tang, Alan Porter, and Boon Tin Chua for valuable comments about the manuscript. This work was supported by grants from the Agency for Science, Technology, and Research (A*STAR) in Singapore. V.C.Y. is an adjunct staff of the Department of Pharmacology, National University of Singapore.

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