Molecular Biology of the Cell
Vol. 17, 3952–3963, September 2006

An Internal EELD Domain Facilitates Mitochondrial Targeting of Mcl-1 via a Tom70-dependent Pathway[D]

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Submitted April 18, 2006; Accepted June 26, 2006
Monitoring Editor: Janet Shaw

Mcl-1 functions at an apical step in many regulatory programs that control cell death. Although the mitochondrion is one major subcellular organelle where Mcl-1 functions, the molecular mechanism by which Mcl-1 is targeted to mitochondria remains unclear. Here, we demonstrate that Mcl-1 is loosely associated with the outer membrane of mitochondria. Furthermore, we demonstrate that Mcl-1 interacts with the mitochondrial import receptor Tom70, and such interaction requires an internal domain of Mcl-1 that contains an EELD motif. A Tom70 antibody that blocks Mcl-1–Tom70 interaction blocks mitochondrial import of Mcl-1 in vitro. Furthermore, Mcl-1 is significantly less targeted to mitochondria in Tom70 knockdown than in the control cells. Similar targeting preference is also observed for the DM mutant of Mcl-1 whose mutation at the EELD motif markedly attenuates its Tom70 binding activity. Together, our results indicate that the internal EELD domain facilitates mitochondrial targeting of Mcl-1 via a Tom70-dependent pathway.

INTRODUCTION

Mcl-1 is one Bcl-2 family member that contains four Bcl-2 homology (BH) domains (BH1–4) and a C-terminal hydrophobic tail. The latter domain was shown to be essential for its targeting to intracellular membranes (Yang *et al*., 1995). Several reports have demonstrated that enforced expression of Mcl-1 delays apoptotic cell death induced by various stimuli, including cytokine withdrawal, exposure to cytotoxic agents (etoposide, calcium ionophore, or UV irradiation), and viral infection (Zhou *et al*., 1997; Chao *et al*., 1998; Cuconati *et al*., 2003). Mcl-1 deficiency results in peri-implantation embryonic lethality (Rinkenberger *et al*., 2000). Conditional knockout mouse models further reveal that Mcl-1 is a critical regulator essential for development and maintenance of T and B lymphocytes, and for ensuring the homeostasis of early hematopoietic progenitors (Opferman *et al*., 2003, 2005). Current models suggest that Mcl-1 may modulate apoptotic cell death via direct interaction with the multidomain proapoptotic Bcl-2 family members, including Bax and Bak, and/or the BH3-only proteins, such as Bim, Puma, and Noxa (Chen *et al*., 2005; Kuwana *et al*., 2005; Willis *et al*., 2005).

The mitochondrion is one major subcellular compartment where the Bcl-2 family members reside and exert their biological functions. Bcl-2 constitutively associates with the outer membrane of mitochondria (MOM) as well as other extramitochondrial membranes (Krajewski *et al*., 1993; Nguyen *et al*., 1993; Lithgow *et al*., 1994; Kaufmann *et al*., 2003). Bcl-X_L is found both in the cytosol and mitochondrial membranes in healthy cells (Hsu *et al*., 1997; Hausmann *et al*., 2000). On stimulation by the apoptotic stimuli, the cytosolic fraction of Bcl-X_L translocates to mitochondria (Hsu *et al*., 1997; Nijhawan *et al*., 2003). Bax is normally cytosolic. But, in response to apoptotic signals, it undergoes a conformational change and translocates to the outer membrane of mitochondria (Hsu *et al*., 1997; Wolter *et al*., 1997; Hsu and Youle, 1998). Conversely, Bcl-w is active while it is loosely associated with mitochondria. During apoptosis, a BH3-only protein binds to its hydrophobic pockets and releases its C-terminal domain for membrane insertion (Wilson-Annan *et al*., 2003). Last, Mcl-1, which functions at an apical step in many apoptotic regulatory programs, is mainly localized to mitochondria, but it is also found to be present in other intracellular compartments, including nucleus and other extramitochondrial membranes (Liu *et al*., 2005; Yang *et al*., 1995). The localization of Mcl-1 to these various subcellular compartments seems to be regulated in a cell state–specific manner (Liu *et al*., 2005). Although the tail-anchoring property of the C-terminal hydrophobic domain (Nguyen *et al*., 1993; Kaufmann *et al*., 2003; Jeong *et al*., 2004) and the *cis*-elements involved in the inducible membrane translocation of some Bcl-2 family members have been extensively characterized (Hsu *et al*., 1997; Wolter *et al*., 1997; Hsu and Youle, 1998; Wilson-Annan *et al*., 2003), the cellular machinery with which this family of proteins is targeted into mitochondria has been much less explored.

Most mitochondrial proteins are encoded by nuclear genes and are synthesized as preproteins in cytosol and later imported into mitochondria via translocation complexes in the outer (TOM) and inner mitochondrial membranes. The TOM machinery consists of two import receptors, Tom20 and Tom70, and a number of other subunits that are ar-

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–04–0319) on July 5, 2006.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

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Abbreviations used: co-IP, coimmunoprecipitation; GP, guinea pig; MOM, outer membrane of mitochondria.

ranged in a tightly bound complex termed the "general import pore" (Pfanner and Geissler, 2001; Endo and Kohda, 2002; Hoogenraad *et al*., 2002; Stojanovski *et al*., 2003). Tom20 binds preproteins with both N-terminal presequences as well as internal targeting signals, whereas Tom70 preferentially binds preproteins with internal targeting information (Brix *et al*., 1997). In this report, we present evidence that Mcl-1 interacts with Tom70 and that such interaction facilitates mitochondrial targeting of Mcl-1.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen

The yeast two-hybrid screen was performed as described previously (Liu *et al*., 2005) except that pBTM116-hMcl-1ΔC27, which encodes a LexA-hMcl-1ΔC27 fusion protein was used as bait to screen a human lymphocyte cDNA library.

Expression Vectors

pCDNA3-HA-hMcl-1, pCMV-Flag-hMcl-1, pCDNA3-hBcl-2, pFLAG-hBid, pCDNA3-FLAG-hBimS, pCDNA3-hBak, and pCDNA3-mBax are mammalian expression vectors for expressing various human (h) or mouse (m) proteins as indicated in the construct names. The expression vectors encoding the hMcl-1ΔC102 or hMcl-1ΔC165 mutants were generated by the insertion of a stop linker (5′-GGTAACTAATTAGGACC-3′) into the XhoI or Asp718 site of the pCDNA3-HA-hMcl-1 vector. The expression vectors encoding all N-terminal truncation mutants of hMcl-1 (ΔN90, ΔN145, and ΔN163) were generated by PCR by using specific forward primers and a common reverse primer, hM-M2, as indicated: hM-M2, 5′-CCCGAAGGTACCGAGAGA-3′; hMΔN90, 5′-GGATCCGCGACCCCCGCGAGG-3′; hMΔN145, 5′-GCGGATCCTTGGTCGGGGAATC-3′; and hMΔN163: 5′-GCGGATCCCCGCCGCCAGCAGA-3′.

The resultant PCR products were restricted with BamHI and KpnI before they were used to replace the corresponding fragment of the pCDNA3-HA-hMcl-1 vector. To construct expression vectors encoding the hMcl-1 mutants (L126A, D127A, and L126A/D127A), standard PCR-assisted mutagenesis-coupled cloning methods were carried out using hM-M2 in combination with the following primers (underlined nucleotides differ from the wild-type [wt] sequence): hM-L126A, 5′-GCCCGAAGAGGAGGCGGACGGGTACGAGC-3′; hM-D127A, 5′-CGAAGAGGAGCTGGCCGGGTACGAGCCG-3′; and hM-L126A/D127A, 5′-CCCGAAGAGGAGGCGGCCGGGTACGAGC-3′.

All constructs containing PCR derived regions were verified by DNA sequencing. pHA-hTom70 is a mammalian expression vector encoding an HA-tagged hTom70. This construct was derived by subcloning the hTom70 cDNA fragment from the pBluescript II SK(+)-KIAA0719 plasmid (a gift from Kazusa DNA Research Institute, Chiba, Japan) into the pcDNA3-HA vector. pGEX4T-Tom70 and pGEX4T-Tom70-R192A are bacterial expression vectors that direct the synthesis of wt or the clamp mutant (R192A) of Tom70 C-terminally fused to the glutathione *S*-transferase (GST) protein. pGST-hTom70N267 and pGST-hTom70ΔN267 are bacterial expression vectors encoding GST-tagged hTom70 mutants containing only or without the N-terminal 267 amino acids, respectively.

Antibodies

Tom70-specific polyclonal antibodies were generated in guinea pigs (GP) by using bacterially produced histidine-tagged hTom70. Another Tom70-specific peptide antibody was raised in rabbits by using a synthetic peptide (QTEVAKKYGLKPPTL) corresponding to amino acid residues 584~598 of hTom70. Both antibodies were affinity purified using specific antigens cross-linked to CNBr-activated Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Other antibodies used in this study include those specific to the hemagglutinin (HA)-tag (clone 12CA5; Roche Diagnostics, Indianapolis, IN); FLAG-tag (clone M2), α-tubulin (both from Sigma-Aldrich, St. Louis, MO), Tom20 (FL-145), Mcl-1 (S-19), Bcl-2 (ΔC21), Bax (N-20) (all from Santa Cruz Biotechnology, Santa Cruz, CA), Bak (Upstate Biotechnology, Lake Placid, NY), calnexin (clone C8.B6; Chemicon International, Temecula, CA), Hsp70 (clone C92F3A-5), and Hsp90 (clone AC88) (both from Calbiochem, San Diego, CA).

In Vitro GST Pull-Down Assays

The GST pull-down assay was carried out essentially as described previously (Liu *et al*., 2005) except that wt or various mutants of Mcl-1 transiently produced in CHOP cells were allowed to bind to bacterially produced GST, GST-hTom70, GST-hTom70N267, GST-hTom70ΔN267, or GST-hTom70-R192A. The bound proteins were analyzed by immunoblotting using anti-HA or hMcl-1 antibody.

Indirect Immunofluorescence and Confocal Microscopy

293T cells, cultured on coverslips, were fixed with 4% (wt/vol) paraformaldehyde and 0.2% (vol/vol) glutaraldehyde in phosphate-buffered saline (PBS) for 20 min. After a brief wash with PBS, cells were blocked and permeabilized in PBS containing 0.5% normal goat serum and 0.2% saponin for another 20 min. The cells were then incubated with affinity-purified GP anti-Tom70 and rabbit anti-Mcl-1 antibody (S-19) in PBS containing 3% bovine serum albumin. After 1-h incubation at room temperature (RT), cells were washed with PBS and incubated for another 30 min at RT with Alexa Fluor 555-conjugated goat anti-GP IgG and Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). After three washes with PBS, stained cells were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) and analyzed with confocal fluorescence microscopy (LSM 510 Meta; Carl Zeiss, Thornwood, NY). In some experiments, cells to be analyzed were first stained with MitoTracker before they were incubated with primary antibodies as described above.

Coimmunoprecipitation (co-IP) of Tom70 and hMcl-1

To analyze the interaction between endogenous Tom70 and hMcl-1, 293T cells were pretreated with 1 mM dithiobis(succinimidyl propionate) (Pierce Chemical, Rockford, IL) or vehicle control (dimethyl sulfoxide [DMSO]) for 30 min at RT before they were lysed in the SHE buffer (250 mM sucrose, 10 mM HEPES-KOH, pH7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate. Cell lysates were immunoprecipitated with control, GP anti-Tom70, or rabbit anti-hMcl-1 antibody. The immune complexes were then analyzed by immunoblotting by using antibodies specific to hMcl-1, Tom70, or Bcl-2. To analyze the interaction of ectopically overexpressed HA-Tom70 and FLAG-hMcl-1, a similar co-IP assay as described above was carried out except that cell lysates were from CHOP cells transiently producing these two tagged proteins (Liu *et al*., 2005) and appropriate control or tag antibodies were used in the subsequent immunoprecipitation and immunoblotting steps.

Subcellular Fractionation

Subcellular fractionation was carried out essentially as described by Nijhawan *et al*. (2003). The resultant three fractions, heavy membrane (HM), light membrane (LM), and cytosol, were analyzed by immunoblotting by using antibodies as indicated in the individual figures.

Opti-Prep Density Gradient Centrifugation

Cells to be analyzed were resuspended in ice-cold SHE buffer before they were broken up by passing through a 25-gauge needle 20 times. After two centrifugation steps at 1020 × *g* for 10 min to remove the nuclei and unbroken cells, the postnuclear lysates (PNLs) were layered on top of a 2.5–27.5% (wt/vol) linear iodixanol gradient (Opti-Prep; Axis-Shield, Oslo, Norway) in buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptine, and 10 μg/ml aprotinin) containing 250 mM sucrose. The gradients were then centrifuged at 200,000 × *g* for 3 h at 4°C in an SW41 rotor. Samples were sequentially collected from the top of the gradients into 20 0.5-ml fractions. The fractions were then analyzed by immunoblot by using antibodies specific to Mcl-1, Tom20 (mitochondria marker), calnexin (endoplasmic reticulum [ER] marker), and Bax (cytosolic marker).

In Vitro Mitochondrial Import Assay

Mitochondria were purified according to a published protocol (Schnaitman and Greenawalt, 1968) except that the C57BL/6J mouse liver was used as a source. The in vitro mitochondrial import assay was then carried out using freshly isolated mitochondria and ^{35}S-labeled proteins (synthesized by the TNT-coupled reticulocyte lysate system; Promega, Madison, WI) essentially as described by Suzuki *et al*. (2002). The imported (pellet; P) and unimported fractions (supernatant; S) were subjected to SDS-PAGE, and specific signals were visualized by fluorography and quantified using a Bioimage Analyzer FLA5000 (Fuji, Tokyo, Japan). In some experiments, the imported fractions (P) were further extracted with 100 mM Na_2CO_3 (pH 11.5 or 10.8) for 30 min on ice and were then centrifuged at 100,000 × *g* for 30 min to separate the alkali-resistant (P) and -extractable (S) fractions. To examine the effect of Tom70 antibodies on the import reaction, mitochondria to be used in the import assay were preincubated with control or anti-Tom70 antibodies at 0°C for 30 min before the ^{35}S-labeled proteins were added to the import system.

Generation of Stable Tom70-Knockdown K562 Cells

To knockdown Tom 70 expression in K562 cells, a Tom70 small interfering RNA (siRNA)-expressing vector (pSNG-siTom70) was constructed by inserting a pair of the Tom70 siRNA target sequence (5′-CCTCTGATGCCATCTCCAC-3′) in a reverse orientation into the pSUPER-neo+gfp vector (Oligo-Engine, Seattle, WA) according to the manufacturer's protocol. K562 cells stably transfected with the control or the pSNG-siTom70 vector were selected in growth medium supplemented with 1 mg/ml G418. Two independent

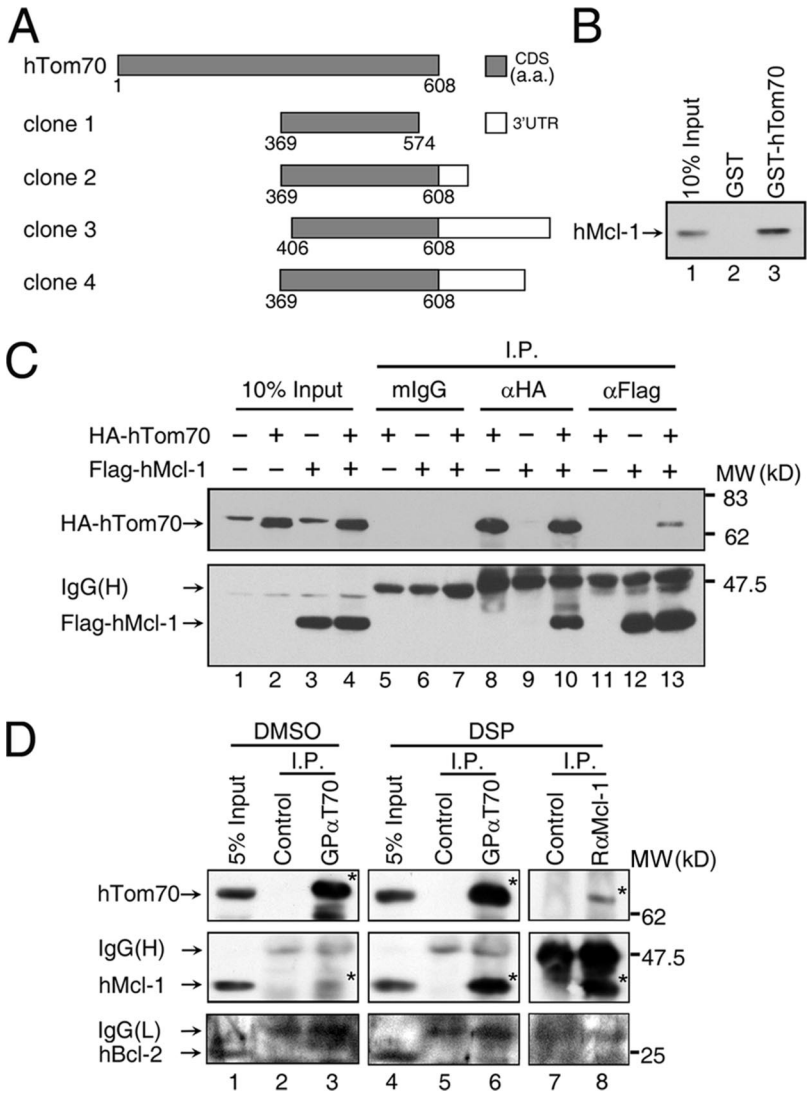


Figure 1. Mcl-1 interacts with Tom70. (A) Schematic representation of four Tom70 cDNA-containing yeast clones fished out by the yeast two-hybrid screen. (B) GST pull-down assay. Bacterially produced GST (lane 2) or GST-hTom70 (lane 3) was allowed to bind to hMcl-1 produced in CHOP cells. Bound proteins and 10% of the lysates added to the binding assay (10% input) were then analyzed by immunoblotting using hMcl-1–specific antibody. (C) Co-IP of ectopically overexpressed HA-hTom70 and Flag-hMcl-1. CHOP cell lysates containing the indicated proteins were immunoprecipitated with control mouse IgG, HA, or FLAG-tag antibody. The immune complexes and one tenth of the lysates used for the immunoprecipitation were analyzed by immunoblotting using HA (top) or FLAG-tag antibodies (bottom). (D) Co-IP of endogenous Mcl-1 and Tom70. Cell lysates prepared from 293T cells pretreated with DMSO (lanes 1–3) or with DSP (lanes 4–8) were immunoprecipitated with control, GP anti-Tom70 antibody (GPαT70) or rabbit anti-hMcl-1 antibody (RαMcl-1). The immune complexes and 5% of those cell lysates used in the immunoprecipitation step were analyzed by immunoblotting with antibody specific to Tom70, hMcl-1, or hBcl-2. Asterisks marked the specific bands recognized by each antibody. IgG (H) and IgG (L) denote the heavy and light chains of the antibodies used in the assay.

clones (K-2 and K-11) with a best Tom70 knockdown efficiency and a pooled mixture of cells transfected with the control vector (Kv) were selected for further analysis.

RESULTS

Mcl-1 Interacts with Tom70

This study was originally aimed to identify cellular factors that might interact with Mcl-1 and modulate its activity. We thus used the yeast two-hybrid approach with hMcl-1 devoid of C-terminal 27 amino acids (hMcl-1ΔC27) as bait. By screening $\sim 9 \times 10^6$ clones from a human lymphocyte cDNA library, we obtained 58 positive clones. Sequence analysis revealed that these 58 clones represent cDNA fragments derived from 18 independent genes, some of which were Bcl-2 family members such as Bax, Bid, and Bik. In this study, we report the characterization of one of these 18 clones which turned out to be the human homologue of the fungal mitochondrial import receptor Tom70 (Alvarez-Dolado *et al.*, 1999; Edmonson *et al.*, 2002). Figure 1A illustrates four independent hTom70 cDNA clones that were fished out from our yeast two-hybrid screen.

The interaction between hMcl-1 and hTom70 was further confirmed by an in vitro GST pull-down assay. As shown in Figure 1B, Mcl-1 protein transiently expressed in CHOP cells (Heffernan and Dennis, 1991) was specifically pulled down by the GST-hTom70 fusion protein, but not by the GST protein alone. Next, we examined the in vivo interaction between ectopically overexpressed hMcl-1 and hTom70 by co-IP assays. In this assay, CHOP cells were transiently transfected with expressing vectors encoding HA-tagged hTom70 (HA-hTom70) and/or FLAG-tagged hMcl-1 (FLAG-hMcl-1). As shown in Figure 1C, FLAG-hMcl-1 specifically occurred in immune complexes precipitated by anti-HA antibody, but not by control IgG, and only under conditions when both FLAG-hMcl-1 and HA-hTom70 were coexpressed in the same transfected cells (Figure 1C, compare lanes 7–10). In a reciprocal experiment, the anti-FLAG antibody also specifically coprecipitated HA-hTom70 (Figure 1C, compare lanes 7 and 11–13). We next examined whether the interaction of Mcl-1 and Tom70 could be observed at endogenous levels of both proteins. For this experiment, a similar co-IP experiment (see *Materials and Methods*) was performed with cell lysates from human embryonic kidney cell line 293T. As shown in Figure 1D, although the GP anti-Tom70 antibody could specifically coprecipitate endogenous hMcl-1 (Figure 1D, compare lane 2 using control with

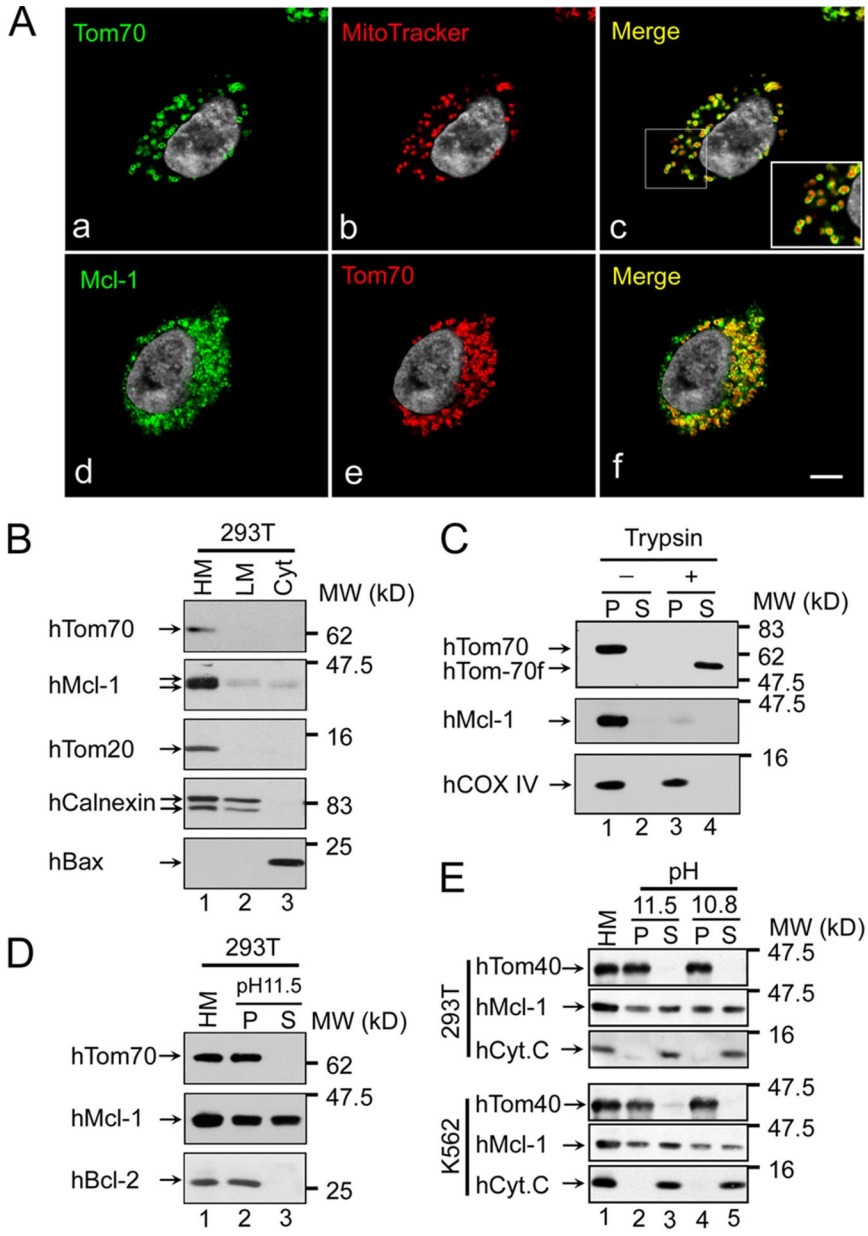


Figure 2. Mcl-1 is loosely associated with MOM. (A) Immunocolocalization of Mcl-1 and Tom70. 293T cells stained simultaneously with antibodies specific to Tom70 (green) and MitoTracker (red) (a–c), or with antibodies specific to Mcl-1 (green) and Tom70 (red) (d–f) were visualized by indirect immunofluorescence and confocal microscopy. Nucleus was counterstained by DAPI (gray). c and f are merged signals for those shown in a and b and d and e, respectively. Inset in C shows the blow-up signals for the region marked. Bar, 5 μm. (B) Immunoblotting of subcellular fractions from 293T cells with various antibodies as indicated. Cyt, cytosol. (C) The isolated mitochondria from the 293T cells remained untreated (lanes 1 and 2) or were subject to trypsin treatment (lanes 3 and 4) as described in *Materials and Methods*, and the resultant P and S fractions were analyzed by immunoblotting with specific antibodies as indicated. hTom-70f denotes a cleaved product of hTom70. (D and E) Same as in C except that isolated mitochondria from 293T or from K562 cells were subject to alkali treatment (pH 11.5 or 10.8) before immunoblotting using antibodies as indicated was carried out.

lane 3 using Tom70 antibody), the amount of hMcl-1 present in the precipitated complex was very low (<1/200 of input proteins; compare lanes 1 and 3). However, under the same co-IP conditions, this Tom 70 antibody could precipitate much more (>50-fold) endogenous hMcl-1 from lysates prepared from 293T cells pretreated with a reversible cross-linking agent DSP (Figure 1D, compare lanes 1 and 3 and lanes 4 and 6). Under the latter conditions, endogenous Tom70 could also be detected in a reverse co-IP experiment using hMcl-1 antibody in the IP step (Figure 1D, lane 8). These results suggest that endogenous Tom70 and Mcl-1 interact with each other. However, their interaction may have been disrupted by the detergent used in the lysis buffer. Notably, under the same conditions, this GP anti-Tom70 antibody failed to coprecipitate Bcl-2, a protein known to target to MOM, from lysates prepared from 293T cells either with or without prior treatment with DSP (Figure 1D, lanes 3 and 6).

Mcl-1 Is Loosely Associated with the Outer Membrane of Mitochondria

Next, we compared the subcellular distributions of hTom70 and hMcl-1. Immunofluorescence analysis by using rabbit anti-Tom70 antibody and a mitochondrion-specific dye MitoTracker revealed that endogenous hTom70 colocalized with MitoTracker (Figure 2A, a–c). Interestingly, under our confocal microscopy settings, the Tom70 antibody gave a doughnut-shaped signal with MitoTracker staining in the center of this structure (see inset in Figure 2A, c). In contrast, in addition to being prominently localized to mitochondria, Mcl-1 is also present in other subcellular compartments (Figure 2A, d–f, and B). Subcellular fractionation analysis further confirmed that Tom70 is exclusively found in the mitochondria-enriched HM fraction, whereas Mcl-1 is predominantly found in the HM fraction with some minor populations residing in the LM and cytosolic fractions (Figure 2B, compare lanes 1–3). Immunoblotting using antibody

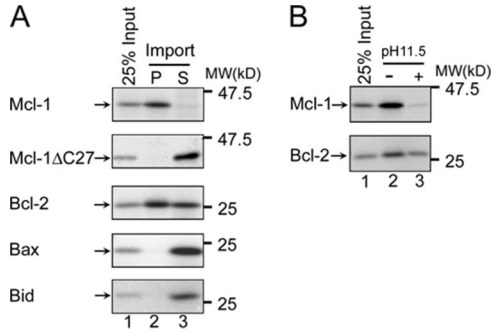


Figure 3. In vitro mitochondrial import of Mcl-1. (A) Cell-free translated S^{35}-labeled proteins as indicated were incubated with isolated mitochondria. After the import assay, the proteins imported into mitochondria (P) or remained in the S fraction S were resolved on SDS-PAGE and visualized by fluorography. Lane 1, 25% of those labeled proteins added to the import assay were loaded on the same gel. (B) Alkali sensitivity of in vitro imported Mcl-1 and Bcl-2. Same as that described for A except that mitochondria reisolated after the import assay were (lane 3) or were not (lane 2) subject to alkali treatment (pH 11.5), and the resultant P fractions were lysed for subsequent analysis.

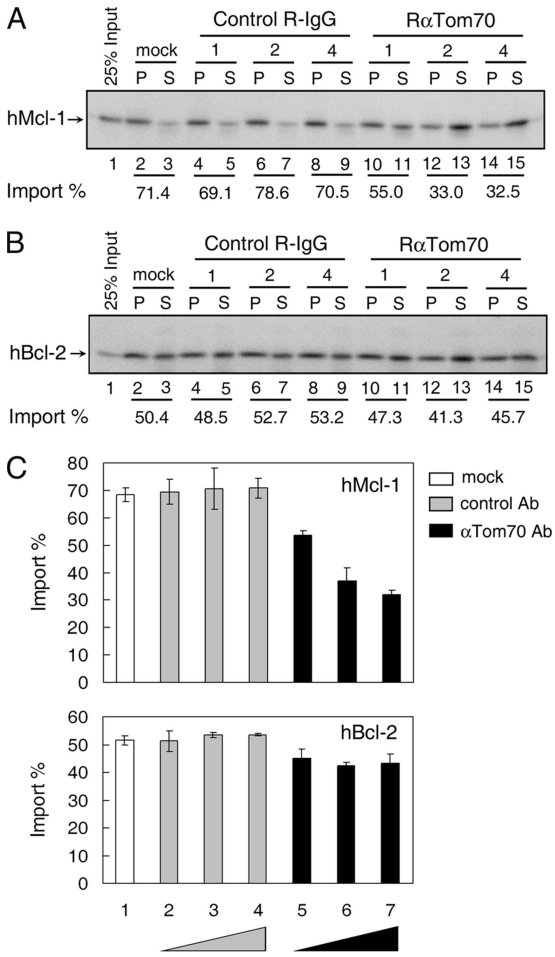


Figure 4. Anti-Tom70 antibody interferes with mitochondrial import of Mcl-1 but not Bcl-2. (A) In vitro mitochondrial import of cell-free translated S^{35}-labeled Mcl-1 was carried out in the presence of 1, 2, or 4 μg of control (lanes 4–9) or affinity-purified rabbit anti-Tom70 antibody (lanes 10–15). After the import reaction, both P and S fractions were analyzed as that described in the legend to Figure 3A. Lanes 2 and 3, an import reaction without any antibody treatment (mock). Import % is counts in the P fraction divided by those in the P plus S fractions. (B) Same as that described for A except that cell-free translated S^{35}-labeled Bcl-2 was used in the import reaction. (C) The average import % of Mcl-1 or Bcl-2 in the presence of increasing amounts (1–4 μg) of control (lanes 2–4) or anti-Tom70 antibody (lanes 5–7) were plotted from the results shown in A and B plus very similar results from one (Bcl-2) or two other (Mcl-1) independent experiments. Error bars are standard deviations of the plotted results.

that recognizes a protein marker specifically localized to ER (calnexin) or mitochondria (Tom20) indicated that the HM fraction analyzed in Figure 2B was slightly contaminated with the LM fraction. However, both the LM and the cytosolic fractions were free of any contaminated mitochondria. We also noticed that Mcl-1 and calnexin did not colocalize with each other in the immunostaining analysis (Supplemental Figure 1), albeit the detection of Mcl-1 in the LM fraction (Figure 2B). These results suggest that Mcl-1 is also localized to some extramitochondrial membranes that are distinct from those of the endoplasmic reticulum, a result that is consistent with that reported by Yang *et al*. (1995).

We next examined whether Mcl-1 is integrated into MOM. For this experiment, isolated mitochondria were treated with or without trypsin before they were lysed for immunoblotting analysis. As shown in Figure 2C, the mitochondrial inner membrane protein, Cox subunit IV, was still detectable in the trypsin-treated mitochondria, i.e., the P fraction shown in Figure 2C (compare lanes 1 and 3), whereas such treatment nearly eliminated the detection of Mcl-1 in the P fraction by the Mcl-1 S-19 antibody (Figure 2C), suggesting that the bulk portion of the Mcl-1 protein was exposed on the cytosolic side of MOM. Of note, under the same conditions, Tom70 was cleaved by trypsin, and a cytoplasmic fragment of ~58–60 kDa was released into the S fraction, a result that is very similar to that reported for the rat homologue of Tom70 (Suzuki *et al*., 2002). Furthermore, like Bcl-2 and Tom40, Tom70 was resistant to alkali extraction (see *Materials and Methods*) after it had integrated into mitochondria (Figure 2, D and E), suggesting that it is also stably integrated into MOM. In contrast, under the same conditions, a great amount of Mcl-1 (>50%) was sensitive to alkali extraction (Figure 2, D and E), indicating that Mcl-1 is loosely associated with MOM.

Mitochondrial Import of Mcl-1 Is Tom70 Dependent

Given that Tom70 is a known mitochondrial import receptor, we next examined whether Tom70 plays a role in the mitochondrial import of Mcl-1. We first isolated mitochondria from the mouse liver and performed an in vitro mitochondrial import assay to address this issue. Under our experimental conditions, cell-free translated Mcl-1 was imported into mitochondria with efficiency ~50–90% (Figure 3A). Also, similar to the case with endogenous proteins (Figure 2, D and E), Mcl-1 imported into the isolated mitochondria was much sensitive to alkali treatment than Bcl-2 imported under the same conditions (Figure 3B). Furthermore, in this assay Bax and Bid, two Bcl-2 family members known to be localized to cytosol in the healthy cells, did not import into isolated mitochondria (Figure 3A, bottom two panels), and neither did the Mcl-1 mutant without the C-terminal transmembrane domain (Mcl-1ΔC27; Figure 3A). Together, these results confirmed the specificity of our in vitro import system. Next, we examined whether an antibody that could block the interaction between Tom70 and Mcl-1 would prevent Mcl-1 from targeting to mitochondria

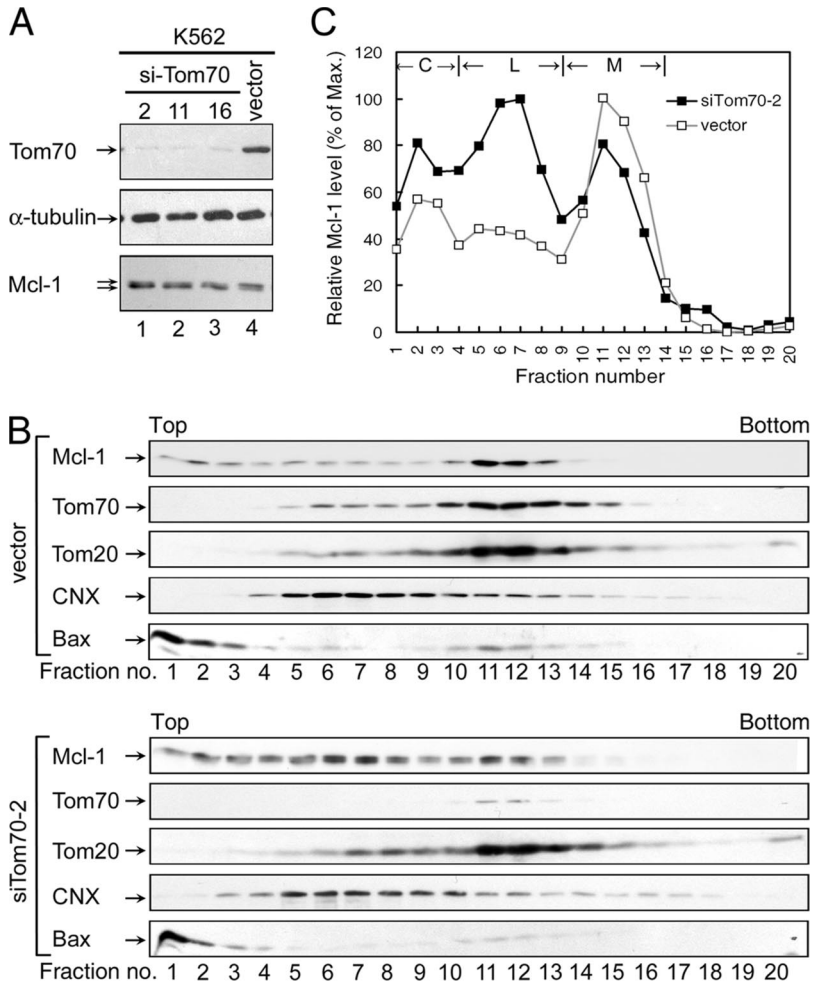


Figure 5. Altered subcellular distributions of Mcl-1 in Tom70 knockdown cells. (A) Tom70 protein levels in K562 cells stably transfected with control (lane 4) or Tom70-specific siRNA expression vectors (clones 2, 11, and 16; lanes 1–3). (B) Fractions from density gradient centrifugation of control (vector) or Tom70 knockdown cells (siTom70-2) were analyzed by immunoblotting with antibody specific to each protein as indicated. (C) Mcl-1 levels of each fraction shown in B were plotted as relative signal to that of the fraction that had the highest amount of Mcl-1. The latter was assigned as 100. Shown here is one representative result from two independent experiments with very similar results. Top and bottom indicate fractions collected from the top to the bottom of the density gradient, respectively.

in the aforementioned in vitro import system. For this experiment, we took advantage of our Tom70 antibody that was raised in rabbits using a peptide corresponding to the C-terminal end of Tom70. Unlike the GP anti-Tom70 antibody that could coimmunoprecipitate Mcl-1 (Figure 1D), the Tom70 peptide antibody raised in rabbits failed to do so (Supplemental Figure 2), suggesting that the latter antibody might interfere with the interaction between Mcl-1 and Tom70. As shown in Figure 4, the affinity-purified rabbit anti-Tom70 antibody, but not the control rabbit IgG, attenuated the in vitro import of Mcl-1 into mitochondria in a dose-dependent manner (Figure 4, A and C). Furthermore, consistent with the lack of interaction between Bcl-2 and Tom70 (Figure 1D), the same Tom70 neutralizing antibody did not exert any significant effect on the in vitro mitochondrial import of Bcl-2 (Figure 4, B and C).

We next investigated whether Tom70 plays a role in mitochondrial import of Mcl-1 in vivo. For this experiment, we first established k562 cells stably expressing a Tom70-specific small hairpin RNA to knockdown endogenous Tom70 levels. As shown in Figure 5A, all three Tom70 knockdown lines whose Tom70 levels were markedly reduced (Figure 5A, top), whereas the levels of an irrelevant protein such as α-tubulin (Figure 5A, middle) were not significantly altered. Interestingly, although the total protein levels of Mcl-1 are similar between the control and the Tom70 knockdown cells (Figure 5A), density gradient fractionation analysis revealed that the subcellular distributions of Mcl-1 in these two groups of cells are significantly different from each other (Figure 5, B and C). In control cells, Mcl-1 was distributed to cytosolic (C; fraction area 1–4), light membrane (L; fraction area 4–9), and mitochondrial (M; fraction area 9–15) fractions with an approximate ratio of 22:29:49 (Figure 5, B and C). However, in Tom70 knockdown cells, Mcl-1 was distributed to these three fractions with a ratio of 24:44:32 (C:L:M) (Figure 5, B and C). Under the same conditions, neither the subcellular distributions of Tom20 nor those of Bax were significantly affected. We could not examine whether knockdown of Tom70 would affect the subcellular distributions of Bcl-2 in K562 cells, because the endogenous Bcl-2 in these cells could not be efficiently detected by the Bcl-2 antibody used in this study. Together, these results suggest that Tom70 plays an important role in mitochondrial targeting of Mcl-1.

The Internal EELD Domain Is Required for Mcl-1 to Interact with Tom70

We next carried out the GST pull-down assays to map the domain of hMcl-1 that interacts with hTom70. For this experiment, we generated a series of mammalian expressing vectors to produce various hMcl-1 mutants with N- or C-terminal deletions (Figure 6A) in the CHOP cells. As shown in Figure 6B (top), deletion of the C-terminal end gradually reduced the Tom70 binding activity of Mcl-1, with ΔC165 retaining ~10–20% of that of the wt protein (see the legend

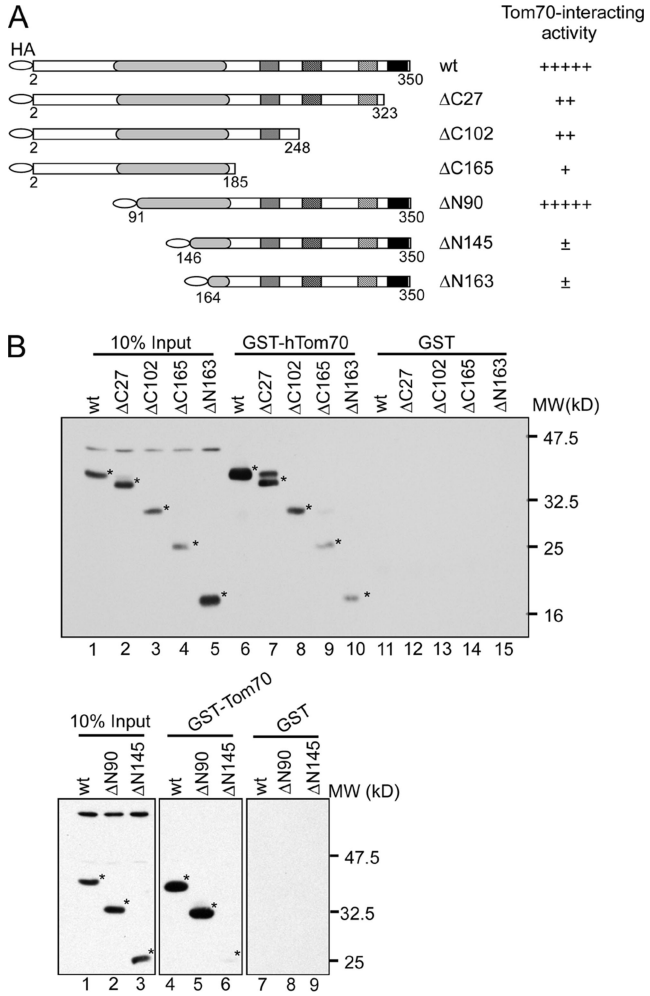


Figure 6. Mapping of Tom70 interacting domain of Mcl-1. (A) Schematic representation of wt and various C- or N-terminal truncation mutants of hMcl-1. All proteins were HA-tagged (oval) at the indicated amino acid position. The Tom70-interacting activity of these proteins as determined by experiments shown in B is summarized as indicated. The plus sign indicates percentage of input proteins bound to the beads precharged with GST-hTom70. +++++, >70%; ++, 15–25%; +, ~10%; −, <1%. (B) GST-pull down assays. wt or mutant Mcl-1 proteins produced in CHOP cells were allowed to bind to beads containing GST or GST-hTom70. Bound proteins were analyzed by immunoblotting using anti-HA antibody. Ten percent of those proteins added to the binding reaction were loaded to the same gel (lanes 1–5, top; lanes 1–3, bottom) and analyzed as that described for the bound proteins. Asterisks mark the respective wt or mutant proteins. An arrowhead points to an unknown form of ΔC27 that interacted with Tom70.

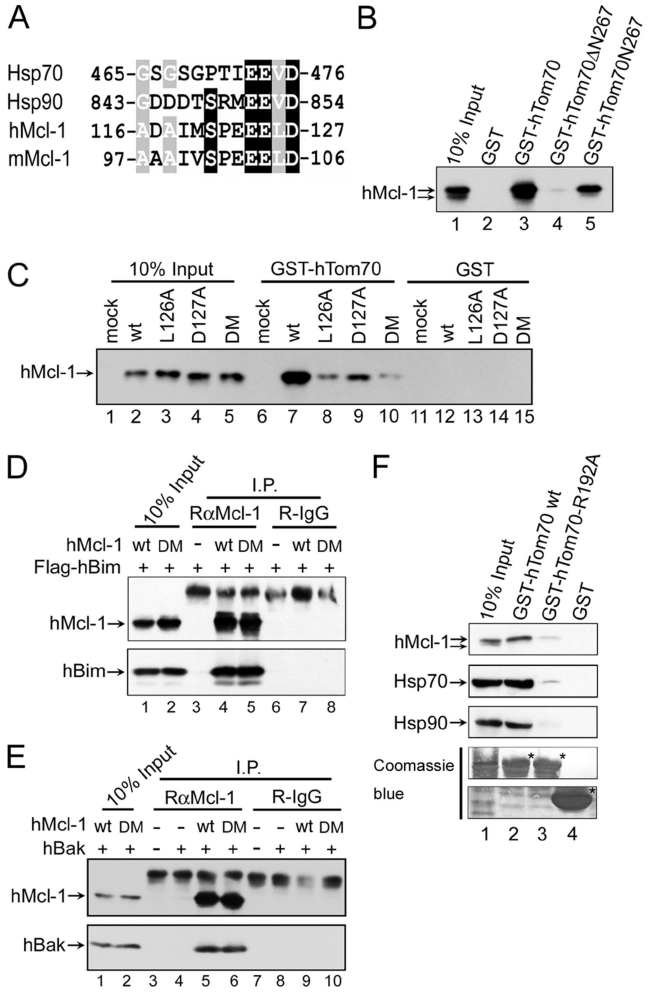
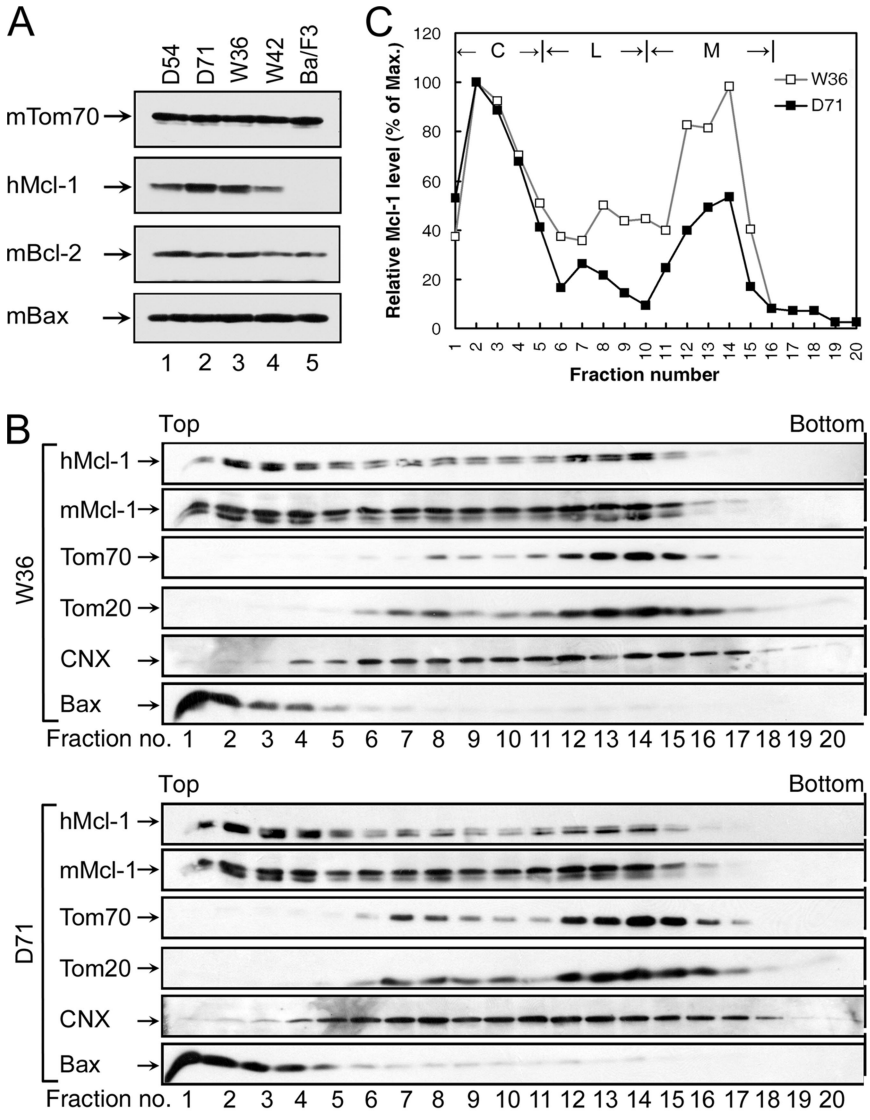


Figure 7. The EELD domain of Mcl-1 interacts with hTom70. (A) Sequence alignment of the EEVD (or EELD)-containing regions from Hsp70, Hsp90, and Mcl-1. (B) The N-terminal fragment of hTom70 (hTom70N267) binds to Mcl-1 better than its C-terminal fragment (hTom70ΔN267). The GST pull-down assay was carried out using hMcl-1 produced in CHOP cells and beads containing GST or GST fusion proteins as indicated. Bound proteins were analyzed by immunoblotting using anti-Mcl-1 antibody. (C) Compromised Tom70 binding activity of the EELD domain mutants of Mcl-1. wt and mutant Mcl-1 as indicated were compared for their abilities to bind GST or GST-hTom70 fusion proteins by the GST pull-down assays. (D and E) The DM mutant of Mcl-1 retains a wt-like binding affinity for Bim and Bak. The co-IP experiments were carried out using lysates from CHOP cells overexpressing the wt or DM mutant of Mcl-1 plus coexpressed hBim (D) or hBak (E). Cell lysates were immunoprecipitated with control (R-IgG) or hMcl-1 antibody (RαMcl-1). Ten percent of the input cell lysates and the immune complexes were analyzed by immunoblotting by using hMcl-1 (top of D and E), FLAG (bottom of D), or Bak (bottom of E) antibodies. (F) The clamp mutant of Tom70 (R192A) has nearly lost its binding activity to hMcl-1. The GST pull-down assay was carried out essentially as that described in the legend to B except that 293T cell lysates were used. The bound proteins were analyzed by immunoblotting by using antibodies specific to hMcl-1, Hsp70, and Hsp90 as indicated. The bottom two panels show the Coomassie blue staining of the GST or the GST fusion proteins (marked by asterisks) immobilized on the beads.

to Figure 6A for definition of the Tom70-interacting activity). Alternatively, although deletion of N-terminal 90 amino acids as in the ΔN90 mutant did not significantly affect Mcl-l's binding to Tom70, further N-terminal deletion down to amino acid residue 145 or 163 nearly abolished Mcl-1's ability to interact with Tom70 (Figure 6B, top, compare lanes 5 and 10; bottom, compare lanes 3 and 6). Although this experiment did not exclude the possibility that the C-terminal hydrophobic tail of Mcl-1 was involved in Mcl-1-Tom70 interaction, it did suggest that the region between amino acid residues 91 and 145 of hMcl-1 was required for such kind of protein–protein interaction. Within this region of Mcl-1, we noticed the presence of an EELD sequence, which is similar to the EEVD motif of two chaperone proteins (Hsp90 and Hsp70) (Figure 7A), that is recognized by two distinct tetratricopeptide repeat (TPR) domains (TPR1 and



D

Experiment	C:L:M ratio wt	C:L:M ratio DM	Difference in percentage of Mcl-1 (wt - DM) localized to mitochondria
1	34 : 24 : 42	51 : 17 : 32	10
2	45 : 26 : 29	60 : 26 : 14	15
3	41 : 27 : 32	47 : 30 : 23	9
4	45 : 27 : 28	62 : 25 : 13	15
			Average 12.3 ± 3.2*

Figure 8. Altered subcellular distributions of the DM mutant of Mcl-1. (A) Protein expression of Ba/F3 cells stably overexpressing wt (clones W36 and W42) or the DM mutant of hMcl-1 (clones D54 and D71). Cell lysates from parental or the indicated clone of Ba/F3 derivatives were analyzed by immunoblotting by using antibodies as indicated. (B and C) Ba/F3 derivatives (clones W36 and D71) were fractionated and analyzed as that described in the legend to Figure 5, B and C. Shown here is one representative fractionation analysis from four independent experiments. (D) Subcellular distribution ratios of Mcl-1 (wt or the DM mutant) from four independent experiments. Numbers underlined represent those from the M fraction. Asterisk, statistically significant, $p = 0.018$.

TPR2A) of the adaptor protein (p60/Hop) of these two chaperones (Scheufler *et al.*, 2000; Brinker *et al.*, 2002). Although the internal EELD sequence of Mcl-1 did not seem to conform to the rule revealed by the structural study where a TPR "clamp" domain and the EEVD motif interaction relies on bonds formed with the carboxylate group on the C-terminal aspartate residue (Scheufler *et al.*, 2000), the following three findings still prompted us to examine whether the EELD motif is essential for Mcl-1 to interact with Tom70: 1) The N-terminal three TPR domains of human Tom70 are structurally similar to the TPR1 and TPR2A domains of p60/Hop (Young *et al.*, 2003). 2) The Tom70 mutant containing the N-terminal three TPR domains as in GST-hTom70N267 bound to Mcl-1 with an affinity that was much higher than the mutant without this N-terminal region (Figure 7B). 3) The Bcl-2 member that does not interact with Tom70, e.g., Bcl-2 (Figure 1D), lacks such a characteristic EELD motif. We therefore generated Mcl-1 mutants with mutations in this motif. As shown in Figure 7C, mutation of either Leu-126 or Asp-127 to alanine markedly attenuated the interaction of Mcl-1 with Tom70 (compare lanes 7–9 in Figure 7C). Mutation of both residues to alanines (L126A and D127A) resulted into a mutant (DM) that lost more than 70% of Mcl-1's binding affinity for Tom70 (Figure 7C, compare lanes 7 and 10; also see Supplemental Figure 3). The wt-like binding activity of the DM mutant to both Bim and Bak as deter-

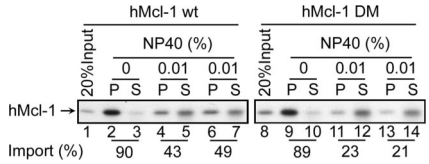


Figure 9. The DM mutant is significantly less targeted to mitochondria than wt Mcl-1 in the in vitro mitochondrial import assay. The in vitro import assay was carried out essentially as that described in the legend to Figure 3, except that in some samples (lanes 4–7 and 11–14) the import buffer was supplemented with 0.01% NP-40 as indicated.

mined by the co-IP assays (Figure 7D and 7E) suggested that the markedly attenuated interaction between the DM mutant of Mcl-1 and Tom70 was not due to a global conformational change of the DM mutant itself. Instead, this result suggests that the EELD motif of Mcl-1 resides in a domain that is critical for Mcl-1 to interact with Tom70.

We next examined whether the TPR clamp domain of Tom70 was involved in the Mcl-1–Tom70 interaction. To address this issue, we investigated whether the clamp mutant of Tom70 (R192A), which failed to bind to Hsp70 and Hsp90 (Young *et al*., 2003), could still interact with Mcl-1. To our surprise, under conditions when GST-Tom70 bound to Mcl-1 quite efficiently, the Mcl-1 binding activity of the GST-fused clamp mutant of Tom70 (GST-R192A) was markedly reduced (Figure 7F, compare lanes 2 and 3), a result that was very similar to the markedly compromised binding of GST-R192A to Hsp70 or to Hsp90 (Figure 7F, middle two panels). Together, our result suggests that the Mcl-1–Tom70 interaction is likely mediated via the internal EELD domain of Mcl-1 and the TPR clamp of the Tom70 molecule, albeit it does not seem to conform to the rule revealed by the structural studies as mentioned previously (Scheufler *et al*., 2000). In contrast, the Tom70 mutant study seems to be inconsistent with that obtained by the yeast-two hybrid screen where Tom70 clones without the N-terminal clamp domain were fished out by Mcl-1. Our best interpretation for these inconsistent results is that other structurally similar TPR motifs present in the core and/or the C-terminal region of Tom70 (Chan *et al*., 2006; Wu and Sha, 2006) may have mediated the Tom70–Mcl-1 interaction in the yeast two-hybrid assay, whose sensitivity apparently differs from that of the co-IP or the GST pull-down assays.

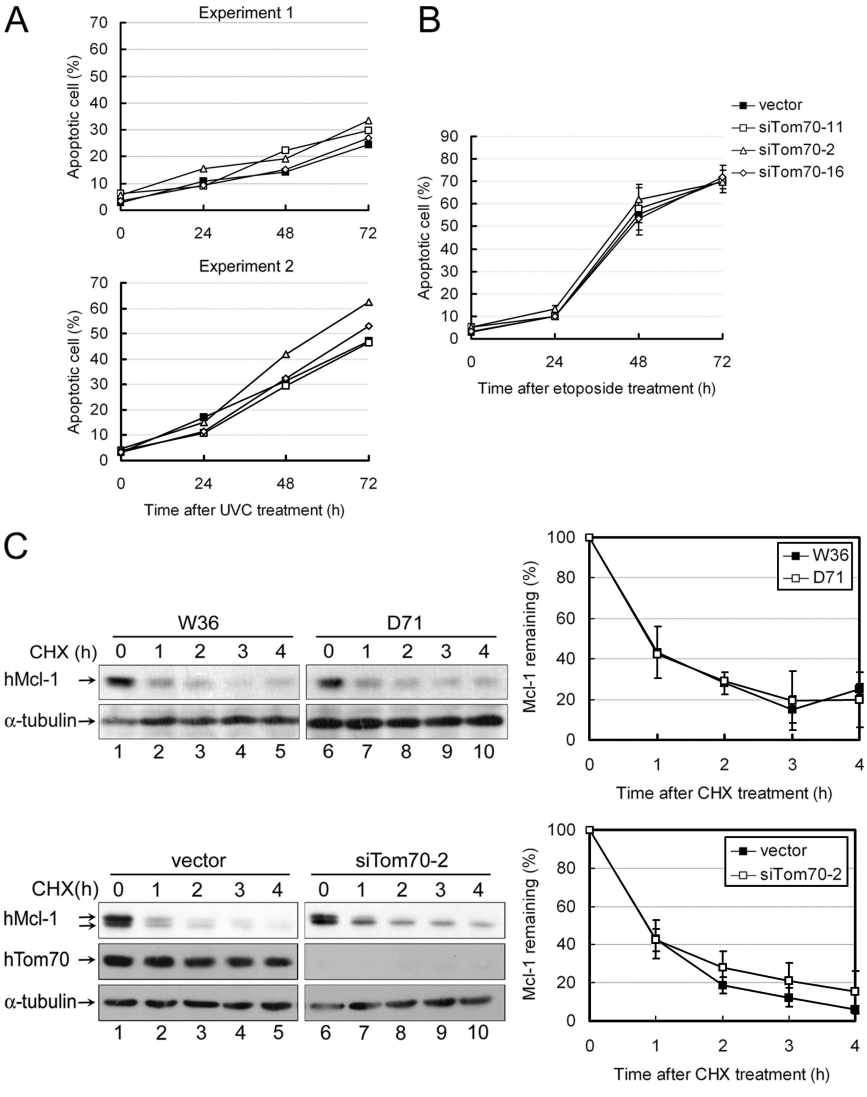


Figure 10. (A and B) Similar apoptosis sensitivity of control and Tom70 knockdown cells. Control (vector) or Tom70 knockdown cells (clones siTom70-2, -16, and -11) were treated with UV (100 mJ/cm^2; A) or etoposide (100 μg/ml; B) for various times as indicated. Apoptotic cells were measured by Annexin V staining assay. (C) Top, protein stability of wt (in clone W36) and the DM mutant of Mcl-1 (in clone D71) is very similar. Cells as indicated were treated with cycloheximide for various times (hours) as indicated before cell lysates were prepared and analyzed by immunoblotting with antibodies specific to hMcl-1 or α-tubulin. Right, turnover kinetics from three independent experiments. (C) Bottom, Mcl-1 stability in control and Tom70 knockdown cells (clone siTom70-2) is very similar. Cells were treated and analyzed as that described in the legend to C, top.

The DM Mutant Is Significantly Less Targeted to Mitochondria

Next, we examined whether the L126A and D127A mutations affect the subcellular localization of Mcl-1. To address this issue, we first established Ba/F3 derivatives stably overexpressing wt (clones W36 and W42) or the DM mutant (clones D54 and D71) of hMcl-1 (Figure 8A). Density gradient fractionation analysis was then used to compare the difference in subcellular distributions of these two proteins (one representative experiment is shown in Figure 8, B and C). Analysis of results from four independent experiments (Figure 8D) indicates that although the intracellular distribution ratios (C:L:M) of Mcl-1 (wt and DM) vary with the culture conditions of each independent experiment, under the same conditions, the DM mutant is consistently less targeted to mitochondria than the wt protein ($12.3 \pm 3.2\%$, $n = 4$, $p = 0.018$). Of note, in both cell clones (W36 and D71), the intracellular distributions of endogenous mMcl-1 were quite similar (Figure 8B), confirming the specificity of this assay. Next, we address the same issue by using another approach, i.e., compare the mitochondrial targeting efficiency of wt and the DM mutant by using the in vitro import assay. The result shown in Figure 9 indicates that the DM mutant was significantly less (compare 21–23% for the DM mutant vs. 43–49% for wt) targeted to mitochondria than the wt protein under assay conditions containing 0.01% NP-40, albeit no significant difference could be observed for these two proteins under our regular in vitro import assay, i.e., without the inclusion of NP-40 in the assay buffer. This latter result is probably due to the sensitivity limit of our assay system where a small difference in the targeting efficiency of two proteins can be differentiated only under a more stringent assay condition. Together, these two different assay methods further support the notion that mitochondrial targeting of Mcl-1 is mediated via a Tom70-dependent pathway.

Similar Apoptosis Sensitivity and Mcl-1 Stability of Control and Tom70 Knockdown Cells

Last, we examined the possibility that the Mcl-1-Tom70 interaction itself has an additional functional role within the cell. To address this issue, we first compared the apoptosis sensitivity of control and Tom70 knockdown cells by treatment of these cells with apoptotic stimuli such as UV and etoposide. The results shown in Figure 10 indicate that knockdown of Tom70 did not significantly alter the apoptosis sensitivity of K562 cells (Figure 10, A and B). We next examined whether Mcl-1–Tom70 interaction may affect Mcl-1 stability. To address this issue, we first compared the protein stability of the wt and the DM mutant of Mcl-1 overexpressed in the Ba/F3 stable lines. As shown in Figure 10C (top), no significant difference in the stability of these two proteins could be observed. We next compared Mcl-1 stability in control and Tom70 knockdown cells. Knockdown of Tom70 resulted in the decrease of the slowest migrating form, and an increase of the faster migrating band of Mcl-1 (Figures 5A and 10C, bottom; the latter band sometimes shows as a doublet in some gel runs). It was technically difficult to completely separate these isoforms and to precisely quantify each one of them; therefore, we quantified them together. As shown in Figure 10C (bottom), again no significant difference in the Mcl-1 stability could be observed between control and the Tom70 knockdown cells ($p > 0.1$, $n = 3$). Collectively, these results suggest that the possibility that the Mcl-1–Tom70 interaction itself is functionally important for Mcl-1 activity is low, at least in the assay systems used in this study. In contrast, considering that Tom70 can mediate mitochondrial import of a few other client proteins in an in vitro import assay (Young *et al*., 2003) and that it can potentially interact with other Bcl-2 family members, such as Bim and Bak (Supplemental Figure 4), the apoptotic response of the Tom70 knockdown cells may not truly reflect the functional aspect of the Tom70–Mcl-1 complex itself. More experiments are required to address this important issue.

DISCUSSION

In this study, we have demonstrated that Mcl-1 interacts with the mitochondrial import receptor Tom70 and such interaction facilitates Mcl-1's import onto mitochondrial membrane. Of note, the Mcl-1 mutant without the C-terminal hydrophobic tail (Mcl-1ΔC27) has a weaker Tom70 binding activity. Furthermore, this mutant cannot target to mitochondria, even though it contains the internal EELD domain. Our results suggest that the C-terminal hydrophobic tail and the internal EELD domain collaborate and direct mitochondrial targeting of Mcl-1.

Three isoforms of Mcl-1 have been noted in the immunoblotting analysis (Yang *et al*., 1995; Chao *et al*., 1998; Liu *et al*., 2005). In the Tom70 knockdown cells, we tend to see much less of the slowest migrating form, suggesting that the slowest migrating form is likely the form that is targeted to mitochondria and possibly via a Tom70-dependent import machinery as well. As mentioned, Mcl-1 is also localized to other extramitochondrial compartments. It remains to be determined what molecular mechanism(s) is responsible for sorting Mcl-1 into various subcellular localizations. Characterization of the identity and functions of various isoforms of Mcl-1 would certainly help address this intriguing question.

Mcl-1, Bcl-2, and Bcl-w are all found to be localized to mitochondria. However, their association with this organelle seems to be different. Bcl-2 is stably integrated into mitochondria, whereas Mcl-1 and Bcl-w are both loosely attached to this organelle. The machinery via which Bcl-w is imported into mitochondria has not been identified. Using the yeast system, the targeting of Bcl-2 into mitochondria was shown to be dependent on Tom20 but not on Tom70 (Motz *et al*., 2002). Furthermore, it was shown that such targeting requires the C-terminal hydrophobic tail of Bcl-2 (Motz *et al*., 2002). In contrast, Shirane and Nakayama (2003) reported that the mitochondrial FK506-binding protein 38 (FKBP38) interacts with Bcl-2 and plays an important role in targeting Bcl-2 to the mitochondrial membrane. An FKBP38 interacting domain was recently mapped to the unstructured loop of Bcl-2 (Kang *et al*., 2005). In this study, we demonstrate that mitochondrial targeting of Mcl-1 is facilitated by Tom70 via the internal EELD motif, which is apparently absent in the Bcl-2 molecule. Although we cannot exclude the possibility that the differential requirement of different import receptors for Bcl-2 and Mcl-1 is due to differences in the assay system (yeast vs. mammalian cells), we favor the interpretation that such differences in use of the import receptor may, at least partially, account for the different physical properties of Mcl-1 and Bcl-2 in association with mitochondria. This latter difference and other known differences in the tissue and subcellular distributions and affinities for various proapoptotic molecules (Chen *et al*., 2005) may all together account for the very different phenotypes of Mcl-1 and Bcl-2 knockout mice (Veis *et al*., 1993; Rinkenberger *et al*., 2000).

In the Tom70 knockdown cells, we observed that more Mcl-1 molecules than those in the control cells are redistrib-

uted to extramitochondrial fractions, albeit the exact subcellular location of these redistributed Mcl-1 molecules is not clear. This result suggests that under conditions when the positive mitochondrial import signal (mediated via the import receptor Tom70) is limiting, Mcl-1 is then randomly sorted to all intracellular locations. This interpretation is further supported by the results from experiment using Mcl-1 mutant (DM) with markedly reduced affinity for Tom70. In the latter study, the DM mutant is significantly less targeted to mitochondria than the wt protein. As mentioned, our results suggest that for optimal targeting to mitochondria the C-terminal hydrophobic and the internal EELD domains of Mcl-1 both need to collaborate with each other. This is in sharp contrast to that reported for Bcl-X_L where the C-terminal transmembrane domain with some flanking basic residues is sufficient to target the linked reporter protein enhanced green fluorescent protein exclusively to mitochondria (Kaufmann *et al*., 2003). It was further proposed that lack of specific mitochondrial targeting ability of the C-terminal hydrophobic tail of both Mcl-1 and Bcl-2 is mainly due to lack of sufficient basicity in this region of protein sequences (Kaufmann *et al*., 2003). Alternatively, FKBP38 was shown to facilitate the mitochondrial import of Bcl-2 (Shirane and Nakayama, 2003) and likely did so via binding to the N-terminal unstructured loop of Bcl-2 (Kang *et al*., 2005). These results suggest that, like with Mcl-1, mitochondrial targeting of Bcl-2 requires the collaboration of two protein domains, one in the N terminus and the other in the C-terminal end. It remains to be determined whether requirement of two domains for mitochondrial targeting is a common property for those membrane proteins without a specific mitochondrial targeting signal in their membrane targeting/anchoring domain. Intriguingly, FKBP38 can also interact with Bcl-X_L and facilitates its mitochondrial targeting (Shirane and Nakayama, 2003). It would be interesting to determine whether the FKBP38 binding domain resides in the C-terminal mitochondrial targeting sequence of Bcl-X_L, because this region of protein alone was shown to be sufficient to confer on Bcl-X_L a mitochondrial localization (Kaufmann *et al*., 2003).

ACKNOWLEDGMENTS

We thank Kazusa DNA Research Institute for providing the full-length hTom70 cDNA, Dr. Shiu-Ming Shih for technical advice on the yeast two-hybrid screen, Dr. Wen Chang for the Bak expression vector, and Dr. Jeffrey J.Y. Yen for helpful discussion. This work was supported in part by an intramural fund from Academia Sinica and by Grant NHRI-EX(91-93)-9119BN from National Health Research Institutes of Taiwan (to H.-F.Y.-Y.).

REFERENCES

Alvarez-Dolado, M., Gonzalez-Moreno, M., Valencia, A., Zenke, M., Bernal, J., and Munoz, A. (1999). Identification of a mammalian homologue of the fungal Tom70 mitochondrial precursor protein import receptor as a thyroid hormone-regulated gene in specific brain regions. J. Neurochem. *73*, 2240–2249.

Brinker, A., Scheufler, C., Von Der Mulbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and Hartl, F. U. (2002). Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70 × Hop × Hsp90 complexes. J. Biol. Chem. *277*, 19265–19275.

Brix, J., Dietmeier, K., and Pfanner, N. (1997). Differential recognition of preproteins by the purified cytosolic domains of the mitochondrial import receptors Tom20, Tom22, and Tom70. J. Biol. Chem. *272*, 20730–20735.

Chan, N. C., Likic, V. A., Waller, R. F., Mulhern, T. D., and Lithgow, T. (2006). The C-terminal TPR domain of Tom70 defines a family of mitochondrial protein import receptors found only in animals and fungi. J. Mol. Biol. *358*, 1010–1022.

Chao, J. R., *et al*. (1998). mcl-1 is an immediate-early gene activated by the granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling pathway and is one component of the GM-CSF viability response. Mol. Cell. Biol. *18*, 4883–4898.

Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., Colman, P. M., Day, C. L., Adams, J. M., and Huang, D. C. (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol. Cell *17*, 393–403.

Cuconati, A., Mukherjee, C., Perez, D., and White, E. (2003). DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. Genes Dev. *17*, 2922–2932.

Edmonson, A. M., Mayfield, D. K., Vervoort, V., DuPont, B. R., and Argyropoulos, G. (2002). Characterization of a human import component of the mitochondrial outer membrane, TOMM70A. Cell. Commun. Adhes. *9*, 15–27.

Endo, T., and Kohda, D. (2002). Functions of outer membrane receptors in mitochondrial protein import. Biochim. Biophys. Acta *1592*, 3–14.

Hausmann, G., O'Reilly, L. A., van Driel, R., Beaumont, J. G., Strasser, A., Adams, J. M., and Huang, D. C. (2000). Pro-apoptotic apoptosis protease-activating factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 or Bcl-x(L). J. Cell Biol. *149*, 623–634.

Heffernan, M., and Dennis, J. W. (1991). Polyoma and hamster papovavirus large T antigen-mediated replication of expression shuttle vectors in Chinese hamster ovary cells. Nucleic Acids Res. *19*, 85–92.

Hoogenraad, N. J., Ward, L. A., and Ryan, M. T. (2002). Import and assembly of proteins into mitochondria of mammalian cells. Biochim. Biophys. Acta *1592*, 97–105.

Hsu, Y. T., Wolter, K. G., and Youle, R. J. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc. Natl. Acad. Sci. USA *94*, 3668–3672.

Hsu, Y. T., and Youle, R. J. (1998). Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. J. Biol. Chem. *273*, 10777–10783.

Jeong, S. Y., Gaume, B., Lee, Y. J., Hsu, Y. T., Ryu, S. W., Yoon, S. H., and Youle, R. J. (2004). Bcl-x(L) sequesters its C-terminal membrane anchor in soluble, cytosolic homodimers. EMBO J. *23*, 2146–2155.

Kang, C. B., Tai, J., Chia, J., and Yoon, H. S. (2005). The flexible loop of Bcl-2 is required for molecular interaction with immunosuppressant FK-506 binding protein 38 (FKBP38). FEBS Lett. *579*, 1469–1476.

Kaufmann, T., Schlipf, S., Sanz, J., Neubert, K., Stein, R., and Borner, C. (2003). Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane. J. Cell Biol. *160*, 53–64.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W., and Reed, J. C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res. *53*, 4701–4714.

Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., and Newmeyer, D. D. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol. Cell *17*, 525–535.

Lithgow, T., van Driel, R., Bertram, J. F., and Strasser, A. (1994). The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. Cell Growth Differ. *5*, 411–417.

Liu, H., Peng, H. W., Cheng, Y. S., Yuan, H. S., and Yang-Yen, H. F. (2005). Stabilization and enhancement of the antiapoptotic activity of mcl-1 by TCTP. Mol. Cell. Biol. *25*, 3117–3126.

Motz, C., Martin, H., Krimmer, T., and Rassow, J. (2002). Bcl-2 and porin follow different pathways of TOM-dependent insertion into the mitochondrial outer membrane. J. Mol. Biol. *323*, 729–738.

Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J., and Shore, G. C. (1993). Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. J. Biol. Chem. *268*, 25265–25268.

Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W., Du, F., and Wang, X. (2003). Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev. *17*, 1475–1486.

Opferman, J. T., Iwasaki, H., Ong, C. C., Suh, H., Mizuno, S., Akashi, K., and Korsmeyer, S. J. (2005). Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. Science *307*, 1101–1104.

Opferman, J. T., Letai, A., Beard, C., Sorcinelli, M. D., Ong, C. C., and Korsmeyer, S. J. (2003). Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. Nature *426*, 671–676.

Pfanner, N., and Geissler, A. (2001). Versatility of the mitochondrial protein import machinery. Nat. Rev. Mol. Cell Biol. *2*, 339–349.

Rinkenberger, J. L., Horning, S., Klocke, B., Roth, K., and Korsmeyer, S. J. (2000). Mcl-1 deficiency results in peri-implantation embryonic lethality. Genes Dev. *14*, 23–27.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000). Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell *101*, 199–210.

Schnaitman, C., and Greenawalt, J. W. (1968). Enzymatic properties of the inner and outer membranes of rat liver mitochondria. J. Cell Biol. *38*, 158–175.

Shirane, M., and Nakayama, K. I. (2003). Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. Nat. Cell Biol. *5*, 28–37.

Stojanovski, D., Johnston, A. J., Streimann, I., Hoogenraad, N. J., and Ryan, M. T. (2003). Import of nuclear-encoded proteins into mitochondria. Exp. Physiol. *88*, 57–64.

Suzuki, H., Maeda, M., and Mihara, K. (2002). Characterization of rat TOM70 as a receptor of the preprotein translocase of the mitochondrial outer membrane. J. Cell Sci. *115*, 1895–1905.

Veis, D. J., Sorenson, C. M., Shutter, J. R., and Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229–240.

Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., and Huang, D. C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev. *19*, 1294–1305.

Wilson-Annan, J., *et al*. (2003). Proapoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity. J. Cell Biol. *162*, 877–887.

Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G., and Youle, R. J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. *139*, 1281–1292.

Wu, Y., and Sha, B. (2006). Crystal structure of yeast mitochondrial outer membrane translocon member Tom70p. Nat. Struct. Mol. Biol. *13*, 589–593.

Yang, T., Kozopas, K. M., and Craig, R. W. (1995). The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. J. Cell Biol. *128*, 1173–1184.

Young, J. C., Hoogenraad, N. J., and Hartl, F. U. (2003). Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. Cell *112*, 41–50.

Zhou, P., Qian, L., Kozopas, K. M., and Craig, R. W. (1997). Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood *89*, 630–643.