

The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity

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Abstract

Proteome analysis of supernatant of isolated mitochondria exposed to recombinant tBid, a proapoptotic Bcl-2 member, revealed the presence of the serine protease Omi, also called HtrA2. This release was prevented in mitochondria derived from Bcl-2-transgenic mice. Release of Omi under apoptotic conditions was confirmed *in vivo* in livers from mice injected with agonistic anti-Fas antibodies and was prevented in livers from Bcl-2 transgenic mice. Omi release also occurs in apoptotic dying but not in necrotic dying fibrosarcoma L929 cells, treated with anti-Fas antibodies and TNF, respectively. The amino acid sequence reveals the presence of an XIAP interaction motif at the N-terminus of mature Omi. We demonstrate an interaction between endogenous Omi and recombinant XIAP. Furthermore we show that endogenous Omi is involved in enhanced activation of caspases in cytosolic extracts.

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Abbreviations: BIR, Baculovirus IAP repeat; DIABLO, direct IAP binding protein with low pI; MALDI, matrix-assisted laser desorption ionization; MBP, maltose binding protein; MS, mass spectrometry;

PSD, post-source decay; Smac, second mitochondria-derived activator of caspase; tBid, truncated Bid; XIAP, X-linked inhibitor of apoptosis protein

Introduction

The central players in the apoptotic pathway are the caspases, a family of cysteinyl aspartate specific proteases. Caspases are activated in response to diverse stimuli and cleave multiple cellular substrates resulting in dismantling of the cell.¹ Due to its destructive nature, caspase activity should be tightly regulated. The main endogenous inhibitors of caspases are members of the inhibitor of apoptosis (IAP) family of proteins such as XIAP (X-linked IAP).² IAP proteins are characterized by the presence of one, two or three baculovirus IAP repeat (BIR) domains. Several IAPs also have a RING-finger domain at their carboxyl terminus. Although some BIR-domain containing IAP proteins such as Bruce and Survivin can regulate cytokinesis and mitotic spindle formation, IAP proteins seem to be mainly involved in the binding and inhibition of active caspases.³ XIAP physically interacts with activated caspases-3, -7 and -9 and blocks the enzymatic activity through its BIR domains.² Recently it was shown that the BIR2 domain and its adjacent N-terminal sequence are essential for binding of caspases-3 and -7,^{4–6} while the BIR3 domain of XIAP interacts with a conserved motif at the N-terminus of the caspase-9 small subunit.⁷ DIABLO/Smac, a mitochondrial protein that is released in apoptosis, may destabilize these complexes.^{8,9} This leads to quenching of the caspase-inhibitory function of XIAP by competitive interaction with the BIR domains.^{7,10–12} After triggering of death receptors such as TNF-R1 and Fas, procaspase-8 is activated resulting in Bid cleavage generating truncated Bid (tBid) that translocates to mitochondria releasing apoptogenic proteins such as cytochrome *c* and DIABLO/Smac.¹³ Studying the mitochondrial release of such proteins during tBid-treatment of isolated mitochondria,¹⁴ we identified the serine protease Omi as a potential new player in this complex interaction between caspases, inhibitors and regulatory proteins. Omi, also named HtrA2, was identified as the mammalian homologue of the bacterial HtrA endoprotease and is highly conserved from bacteria to mammals.^{15–17} The mammalian orthologue HtrA2/Omi is upregulated during renal ischemia-reperfusion¹⁶ and is putatively involved in cell growth regulation.¹⁵ Although reported as being localized either within the endoplasmic reticulum¹⁶ or the nucleus,¹⁷ we identified Omi as a protein released *in vitro* from isolated mitochondria treated with

recombinant tBid.¹⁴ This translocation could be confirmed in apoptotic models *in vivo* and in a fibrosarcoma cell line. This mitochondrial release of Omi was completely prevented by Bcl-2. Moreover, under necrotic cell death conditions no such translocation is observed. The exposed N-terminal sequence of mature Omi contains a conserved XIAP-interaction motif.⁷ We could demonstrate that Omi interacts with recombinant XIAP, preventing the caspase-inhibitory function of the latter.

Results

tBid is required and sufficient for the release of Omi from isolated mitochondria

In order to study tBid-mediated release of proteins from mitochondria in a systematic way, we performed a proteome analysis on supernatant of isolated mitochondria treated with purified recombinant tBid.¹⁴ One of the proteins identified by mass spectrometry and PSD analysis¹⁸ was the serine protease Omi (Figure 1). Although Omi has a theoretical

molecular weight of 49 kDa, the protein that is identified in mitochondria has a size of 37 kDa as the N-terminal presequence is probably cleaved off after mitochondrial transport producing mature Omi (Figure 1D). This N-terminal presequence contains a mitochondrial targeting sequence as predicted by using the MITOPROT computational method.¹⁹ The presence of this serine protease was obvious in supernatant of purified mitochondria exposed to tBid, as shown by Western blotting (Figure 2A). The release of Omi was completely prevented in mitochondria from Bcl-2 transgenic mice at a concentration of tBid of 10 ng, corresponding to 6.7 nM (Figure 2A). As caspase-8 cleaves endogenous Bid under apoptotic conditions,^{20,21} we prepared cytosols from untreated L929sAhFas cells and exposed these extracts to recombinant caspase-8. When incubated with purified mitochondria, this activated cytosol was able to induce the release of Omi (Figure 2B). Immunodepletion of Bid from L929sAhFas cytosol activated with recombinant caspase-8 completely abrogated the release of this protease (Figure 2B) indicating that endogenous levels of Bid are sufficient and essential for Omi release from mitochondria. Identical results were obtained for the release of cytochrome *c* (Figure 2B).

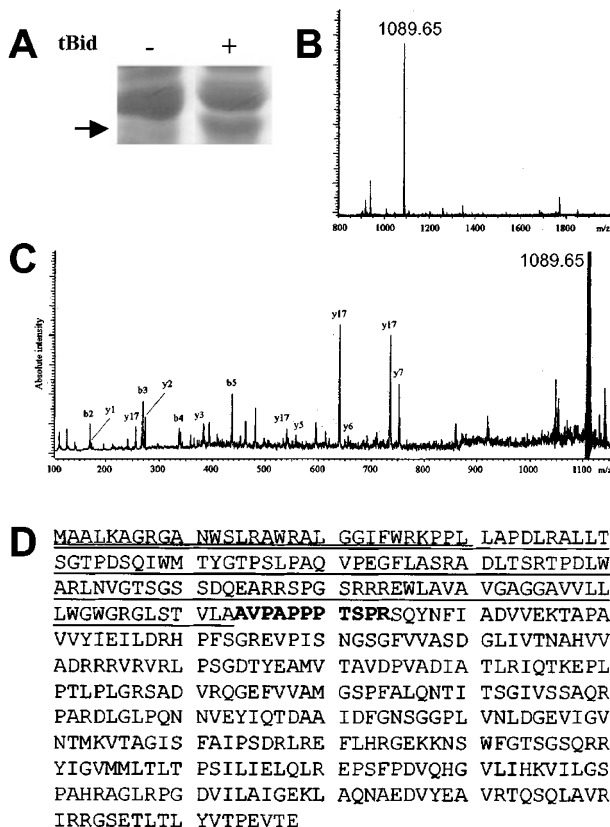


Figure 1 Identification of Omi. (A) A mitochondrial preparation of one mouse liver treated with tBid was subjected to 15% SDS-PAGE and stained with Coomassie Brilliant Blue. The arrow indicates a 37 kDa protein band released from mitochondria. (B) MALDI-MS spectrum of peptides present in the HPLC-fraction from trypsin digests of the 37 kDa band. (C) MALDI-PSD analysis of the peptide with a mass of 1089.65 Da present in the spectrum of (B) led to the identification of the mouse Omi protein. (D) Amino acid sequence of Omi protein. The N-terminal presequence (underlined) contains a mitochondrial targeting sequence (double underlined). The tryptic peptide identified in (C) is highlighted in bold

Omi translocates from mitochondria to the cytosol during apoptosis

The observation that isolated mitochondria release Omi when treated with proapoptotic tBid could be confirmed *in vivo* in a murine model of experimental lethal hepatitis. Administration of agonistic anti-Fas antibodies to mice induced the release of both Omi and cytochrome *c* from the mitochondria to the cytosol. This mitochondrial release was completely absent in Bcl-2 transgenic mice (Figure 2C). L929sAhFas cells were shown to die either by apoptosis when stimulated with anti-Fas, or by necrosis when stimulated with TNF,²² so both cell death pathways occur in the same cellular context depending on the extracellular stimulus. Omi release into the cytosol was restricted to cells that die by apoptosis when exposed to anti-Fas, as was shown previously for cytochrome *c*.²³ No Omi nor cytochrome *c* release was seen in necrotic dying cells when stimulated with TNF (Figure 2D). This observation is related with the absence of tBid generation in necrotic cell death (Figure 2D), illustrating once more the profound difference in the functional role of mitochondria in both ways of cell death.

Omi binds to XIAP and promotes caspase activity

After transport into the mitochondria, the N-terminal 133 amino acid peptide of Omi is cleaved off generating the mature protein.²⁴ Sequence comparison of the N-terminus of the mature mouse and human Omi protein revealed the presence of the conserved XIAP BIR3-interaction motif AVPA and AVPS, respectively. This conserved IAP-binding motif is also seen at the N-terminus of the small subunit of mature caspase-9 and mature DIABLO/Smac (Figure 3A) and it was also described for the *Drosophila* proteins Reaper, Hid and Grim.⁷ To determine if released ~37 kDa Omi can

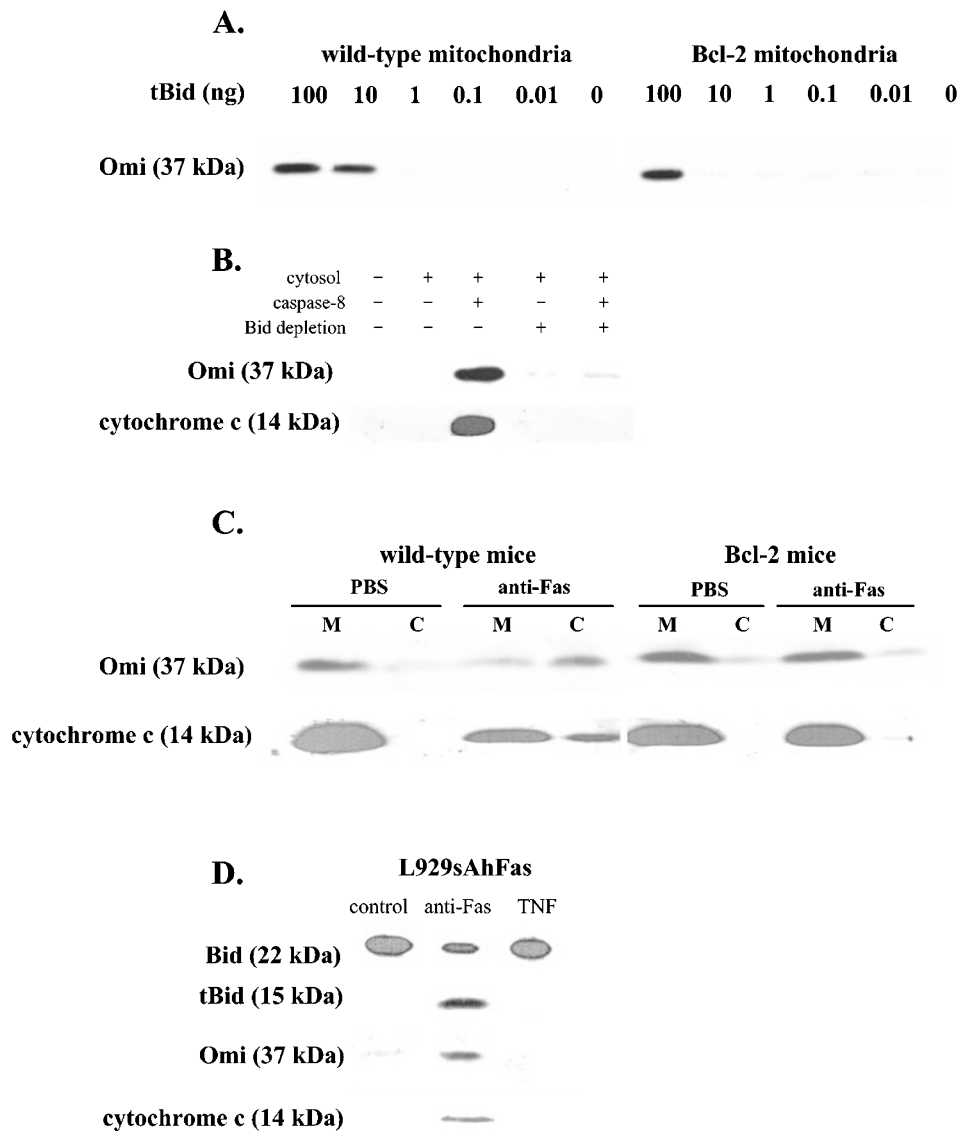


Figure 2 tBid is sufficient and required for mitochondrial Omi release. (A) tBid was incubated with purified liver mitochondria from wild-type and Bcl-2 transgenic mice. Supernatants were separated from the mitochondrial pellets by centrifugation and subjected to SDS-PAGE, followed by immunoblotting with anti-Omi antibody. (B) Endogenous Bid was immunodepleted from L929sAhFas cytosol activated with recombinant caspase-8 (1 μ g) prior to incubation with mitochondria, as indicated. Supernatants were isolated and subjected to SDS-PAGE followed by immunoblotting with anti-Omi and anti-cytochrome *c* antibodies. (C) Wild type and Bcl-2 transgenic mice were intravenously injected with PBS or agonistic anti-Fas antibody and liver mitochondria were prepared as described in Materials and Methods. 100 μ g of mitochondrial (M) and cytosolic (C) lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-Omi or anti-cytochrome *c* antibodies. (D) Non-treated L929sAhFas control cytosol, anti-Fas antibody-induced (250 ng/ml) L929sAhFas apoptotic cytosol and mTNF-induced (10 000 U/ml) L929sAhFas necrotic cytosol were prepared using digitonin lysis buffer and immunoblotted with anti-Omi antiserum and anti-cytochrome *c* antibody. To monitor Bid proteolysis total lysates were prepared

bind to XIAP, we performed an *in vitro* interaction assay in which control and tBid-induced mitochondrial supernatants were incubated with either maltose binding protein (MBP) or with the N-terminal MBP-XIAP fusion protein. Following pull down of the protein complexes with amylose beads, Western blotting revealed the presence of Omi in case of MBP-XIAP. No Omi was found in the condition using MBP protein alone as a control for aspecific binding to MBP and the amylose beads (Figure 3B). It has been reported that DIABLO/Smac disrupts the interaction between processed caspase-9 and

BIR3-XIAP leading to the activation of downstream executioner caspases.⁷ The same could be true for Omi. Therefore, we examined the ability of the tBid-induced mitochondrial supernatant to promote activation of endogenous caspases in L929sAhFas cytosol. This cytosol contains high levels of XIAP (data not shown). tBid-induced mitochondrial supernatant could induce the activation of endogenous executioner caspases demonstrated by an increase in DEVDase activity (Figure 4B). To test whether Omi was involved in the generation of this caspase activity,

A.

mouse Omi	A V P A P P P T S P R S Q
human Omi	A V P S P P P A S P R S Q
mouse casp-9 p12	A V P Y Q E G P R P L D Q
human casp-9 p12	A T P F Q E G L R T F D Q
mouse DIABLO/Smac	A V P I A Q K S E P H S L
human DIABLO/Smac	A V P I A Q K S E P H S L

B.

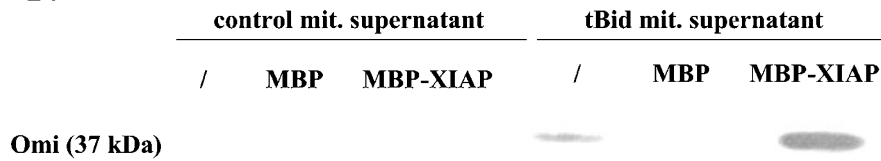


Figure 3 Omi interacts with XIAP (A) Alignment of the N-terminal sequences of mouse and human Omi, caspase-9 p12 subunit and DIABLO/Smac. The XIAP-binding motif is highlighted in bold. (B) Control and tBid-induced mitochondrial supernatants were left untreated, incubated with MBP control or MBP-XIAP as indicated. Protein complexes were isolated with amylose beads and subjected to SDS-PAGE followed by immunoblotting with anti-Omi antibody

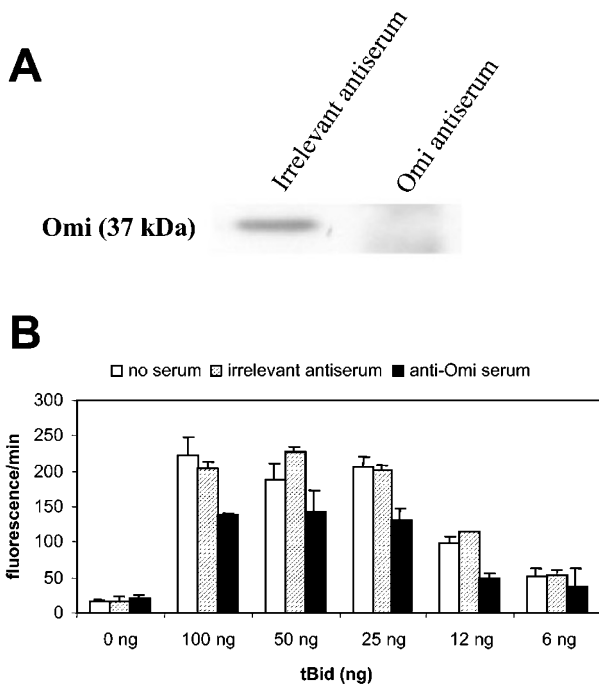


Figure 4 Omi release leads to enhanced caspase activity (A) Immunoblotting with anti-Omi antiserum of tBid-induced mitochondrial supernatant after depletion of Omi with anti-Omi serum. As a control the supernatant was incubated with an irrelevant antiserum. (B) 50 μ g L929sAhFas cytosol was incubated with mitochondrial supernatant, induced with different amounts of tBid as indicated. tBid-induced mitochondrial supernatant was left untreated or immunodepleted with anti-Omi serum. As a control an irrelevant antiserum was used. Caspase activity was measured using Ac-DEVD-amc as a substrate

Omi was immunodepleted from the mitochondrial supernatant. This resulted in reduced DEVDase activity. An analogous treatment with irrelevant antiserum had no effect

(Figure 4A,B). This suggests that Omi plays a proapoptotic role by enhancing caspase activity.

Discussion

Mitochondria are now recognized as central players in the control of cell death mainly through the release of several proapoptotic intermembrane space proteins.²⁵ These proteins include cytochrome *c*,²⁶ apoptosis inducing factor,²⁷ endonuclease *G*^{28,29} and the recently identified DIABLO/Smac.^{8,9} We have reported on the identification of proteins released from isolated mitochondria treated with purified recombinant tBid. One of these proteins was identified as Omi by mass spectrometry and PSD analysis.¹⁴ Omi was recently described as a member of a novel family of mammalian serine proteases homologous to the bacterial HtrA gene product. This serine protease has been described in a wide range of bacteria and eukaryotes and is evolutionary well conserved.¹⁵⁻¹⁷ Bacterial HtrA was shown to act as a chaperone below 28°C and as an active protease above 37°C.³⁰ In mammals, HtrA2/Omi is involved in the degradation of aberrantly folded proteins during conditions of cellular stress such as endoplasmic reticulum stress, heat shock and ischemia-reperfusion,^{16,17} but was also proposed to play a role in cell growth regulation.¹⁵ Although originally reported as localized in the endoplasmic reticulum¹⁶ or the nucleus,¹⁷ we detect Omi in isolated mitochondria with an apparent molecular weight of 37 kDa. The precursor form of Omi has a theoretical molecular weight of 49 kDa. This precursor form contains a mitochondrial localization signal, as predicted using a computational model.¹⁹ During mitochondrial import the first 133 N-terminal amino acids are removed generating mature Omi protein.²⁴ This processing leads to the N-terminal exposition of a conserved AVPA sequence, which can allow interaction with the BIR3-domain in XIAP. A similar interaction was previously described for DIABLO/Smac,

caspace-9 and the *Drosophila* proteins Hid, Grim and Reaper.⁷ We could confirm this predicted binding by pull down experiments using recombinant MBP–XIAP and revelation with anti-Omi antiserum. Recently, structure/function analysis of the Omi–XIAP interaction showed that binding could occur both with the BIR2 or the BIR3 domain.³¹ Besides its interaction with XIAP, we showed that the release of Omi could also increase caspace activity in L929sAhFas cytosol.

Omi interacts with XIAP in a similar way as DIABLO/Smac and both are of mitochondrial origin. However, what could be the difference between both proteins and what could be the role of the redundant mechanism of XIAP regulation? A possible clue might be the differential expression pattern of both proteins. Northern analysis showed that DIABLO/Smac mRNA was most abundant in heart, liver, kidney and testis but expression was not readily detectable in skeletal muscle, lung, thymus and brain.^{8,9} Omi mRNA, however, is expressed ubiquitously.^{16,17} However, another important feature that differentiates Omi from DIABLO/Smac is its serine protease activity. Although no specific activation mechanism or functional substrates were reported so far, it seems that Omi is strongly cytotoxic depending on its catalytic serine while overexpression of DIABLO/Smac is not.³¹ Overexpression of Omi causes an atypical caspace-independent cell death.³¹ Although the cytotoxic activity of a protease after transient overexpression may not be relevant, it is suggestive that Omi may have a bifunctional potential: one as inhibitor of XIAP, propagating caspace cascades, and another one as a serine protease, propagating atypical caspace-independent cell death.³¹ Some studies described a role for serine proteases in the apoptotic cell death process.^{32,33} Also in necrosis, which is generally considered as a caspace-independent process, serine proteases may be involved.²² However, the implication of mitochondrial Omi in necrosis is not very likely in view of our data that demonstrate that during TNF-induced necrosis in L929sAhFas cells no release of Omi could be observed. This does not exclude that the serine protease Omi at other subcellular localizations, such as endoplasmic reticulum¹⁶ or nucleus¹⁷ may be involved. To conclude, our data show that Omi is released from mitochondria during apoptosis, interacts with XIAP and promotes caspace activation. Although its catalytic site seems to be essential for its cell death promoting activity as was shown in overexpression experiments,³¹ the relevance of its protease activity is still unclear. Identification of possible substrates and inhibitors of Omi and its mechanism of activation will be of crucial importance.

Materials and Methods

Animals, cells and reagents

C57BL/6 wild type and liver-specific Bcl-2 transgenic mice³⁴ were used at the age of 7–12 weeks. Monoclonal hamster anti-mouse Fas antibody Jo2 (Pharmingen, San Diego, CA, USA) was dissolved in endotoxin-free PBS and injected intravenously (15 µg in 200 µl per mouse). Livers from sacrificed mice were homogenized on ice (5 mM KH₂PO₄ Ph 7.4, 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS).

L929sAhFas cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), L-glutamine (0.03%) and fetal calf serum (10%). Cells were left untreated, stimulated with 250 ng/ml agonistic anti-human Fas antibody (clone 2R2, Cell Diagnostica, Menster, Germany) for 30 min to 2 h or with 10 000 U/ml recombinant murine TNF (produced in our laboratory by Alex Raeymaeckers) for 2–7 h. Cells were washed in ice-cold PBS and permeabilized in 0.02% digitonin (Boehringer Mannheim GmbH, Mannheim, Germany) dissolved in cell free system buffer (CFS) (10 mM HEPES–NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂ 5 mM pyruvate, 0.1 mM PMSF, 1 mM dithiothreitol) and left on ice for 1 min. This treatment allows selective lysis of the outer cell membrane without affecting the organelle membranes. Lysates were cleared by centrifugation at 20 000 × g for 10 min and stored at 4°C.

Expression and purification of recombinant proteins

Murine tBid (residues 60–195) with a tag of six histidines at the N-terminus was cloned in the pLT10 vector and expressed in *Escherichia coli*. The protein was recovered in the soluble bacterial fraction and purified by cobalt-immobilized metal affinity chromatography (TALON[®], Clontech, Palo Alto, CA) using the manufacturer's protocol. Active recombinant murine caspace-8 was expressed and purified by a similar method.³⁵ XIAP was cloned in frame to the maltose binding protein (MBP) coding region on the pMAL-c2X bacterial expression vector (New England Biolabs, Beverly, MA, USA). Bacteria were grown for 16 h with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 20°C and sonicated in 20 mM Tris–HCl pH 7.5 and 100 mM NaCl supplemented with complete EDTA-free protease inhibitor (Roche, Mannheim). SDS–PAGE, Coomassie brilliant blue staining and Western analysis showed the presence of the MBP–XIAP fusion protein in the soluble fraction. As a control sonicated bacteria containing pMAL–c2X and expressing MBP were used.

Isolation of murine liver mitochondria

Livers of wild-type and Bcl-2 transgenic mice were homogenized and mitochondria were prepared as described earlier,¹⁴ resuspended in CFS buffer, kept on ice and used within 1 h after preparation.

MALDI–PSD protein identification

Identification of Omi from tBid-induced mitochondrial supernatant was performed as described previously.¹⁴

Western blot analysis of tBid-induced release of mitochondrial proteins

Intact mouse liver mitochondria equivalent of 40 µg protein were incubated at 37°C in 100 µl CFS buffer for 20 min with various reagents as indicated in the figure legends. The supernatants were separated from the mitochondria by centrifugation at 20 000 × g for 10 min at 4°C. One fifth of the supernatant was subjected to 15% and 12.5% SDS–PAGE followed by Western blotting with anti-cytochrome c antibody (clone 7H8.2C12, Pharmingen, CA, USA) or an antibody raised against Omi, respectively. Blots were visualized with the chemiluminescence NEN Renaissance method (Du Pont, Wilmington, DE, USA) after incubation of membranes with secondary antibody coupled to horseradish peroxidase (Amersham Pharmacia Biotech, Rainham, UK).

Immunodepletion and coprecipitation experiments

For immunodepletion of tBid, L929sAhFas digitonin lysates were precleared 3 times by incubation for 5 min with 20% protein G beads (Amersham Pharmacia Biotech, Rainham, UK) and incubated with anti-Bid antibody (10 $\mu\text{g} \times 10^6$ cells) (R&D Systems Inc., Minneapolis, MN, USA) for 1.5 h at 4°C. The antibody complexes were captured with protein G beads (20% slurry) for 30 min at 4°C and removed by centrifugation. The resulting Bid-depleted supernatant was used in the reconstitution experiment with purified liver mitochondria. For immunodepletion of Omi from tBid-induced mitochondrial supernatant a comparable procedure was followed using Tris-Acryl protein A beads (Pierce, Rockford, IL, USA). Pull-down experiments of Omi, control and tBid-induced mitochondrial supernatants were incubated with bacterial sonicate containing 10 μg MBP and MBP-XIAP in CFS buffer for 1.5 h at 4°C. MBP-interacting proteins were pulled down by amylose resin (New England Biolabs, Beverly, MA, USA), beads were isolated by centrifugation, washed three times in CFS buffer and boiled in laemli buffer. After centrifugation, the supernatant was separated on 12.5% SDS-PAGE followed by immunoblotting with anti-Omi antibody.

DEVD-amc cleavage assay

L929sAhFas cytosol, MBP-XIAP and tBid-induced mitochondrial supernatant were coincubated with 50 μM acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-amc) (Peptide Institute Inc., Osaka, Japan) in 150 μl CFS buffer. The release of fluorescent 7-amino-4-methylcoumarin was measured for 50 min at 2-min intervals by fluorospectrometry at 360 nm excitation and 480 nm emission wavelength and the maximal rate of increase in fluorescence was calculated ($\Delta\text{fluorescence}/\text{min}$) (Cytofluor, PerSeptive Biosystems, Cambridge, MA, USA).

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