

A matrix-assisted laser desorption ionization post-source decay (MALDI-PSD) analysis of proteins released from isolated liver mitochondria treated with recombinant truncated Bid

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Abstract

A crucial event in the process of apoptosis is caspase-dependent generation of truncated Bid (tBid), inducing release of cytochrome *c*. In an *in vitro* reconstitution system we combined purified recombinant tBid with isolated liver mitochondria and identified the released proteins using a proteomic matrix-assisted laser desorption ionization post-source decay (MALDI-PSD) approach. In order to meet physiological conditions, the concentration of tBid was chosen such that it was unable to induce cytochrome *c* release in mitochondria derived from liver-specific Bcl-2-transgenic mice. Several mitochondrial proteins were identified to be released in a tBid-dependent way, among which cytochrome *c*, DIABLO/Smac, adenylate kinase 2, acyl-CoA-binding protein, endonuclease G, polypyrimidine tract-binding protein, a type-I RNA helicase, a WD-40 repeat-containing protein and the serine protease Omi. Western blotting confirmed the absence of adenylate kinase 3, a matrix mitochondrial protein. These results demonstrate that a physiologically relevant concentration of tBid is sufficient to induce release of particular intermembrane mitochondrial proteins belonging to a broad molecular-mass range.

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Keywords: apoptosis; caspase; MALDI-PSD; mitochondria; tBid

Abbreviations: AK, adenylate kinase; ER, endoplasmic reticulum; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PSD, post-source decay; PTB, polypyrimidine tract binding; tBid, truncated Bid

Introduction

It has become clear that mitochondria play a central role in the process of cell death. These organelles are targets for proapoptotic and antiapoptotic pathways. Mitochondria participate in the apoptotic process by releasing factors that modulate the activation of caspases. In this respect, release of cytochrome *c* is required for formation of the Apaf-1/procaspase-9 apoptosome complex in the presence of (d)ATP.^{1,2} The recently identified DIABLO/Smac unlatches the inhibitory activity of XIAP on caspase-3, caspase-7 and caspase-9,^{3–8} favoring execution of the apoptotic pathway. Also during necrotic cell death, mitochondria are involved in the production of reactive oxygen species,⁹ but no release of cytochrome *c* has been observed.¹⁰

Many different apoptogenic stimuli impinge on the specific proteolysis of Bid, a proapoptotic BH3-only member of the Bcl-2 family.¹¹ Bid is proteolytically cleaved by caspase-8, generating 15-kDa truncated Bid (tBid),^{12,13} by granzyme B, leading to 14-kDa tBid,^{14–16} or by lysosomal proteases.¹⁷ In the prototype CD95 (Fas) apoptotic pathway, ligand binding induces formation of a death-inducing signaling complex, consisting of the Fas-associated death domain protein and procaspase-8.¹⁸ The molecular link between these receptor-mediated events and mitochondria is provided by caspase-8-mediated proteolysis of Bid.^{12,13} tBid translocates to the mitochondria and induces cytochrome *c* release, a process involving the proapoptotic Bcl-2 members Bax and Bak.^{19–22} Bid-deficient mice are resistant to anti-Fas-induced hepatocyte apoptosis,²³ which emphasizes the importance of this death domain receptor/mitochondrial connection. In such hepatocytes, anti-Fas-induced mitochondrial dysfunction is delayed, no cytochrome *c* is released and activation of downstream effector caspases is reduced. Antiapoptotic Bcl-2 and Bcl-X_L proteins do not block cleavage and relocalization of tBid, but can prevent tBid-induced cytochrome *c* release.^{24,25}

Although cytochrome *c* is the best characterized protein released by mitochondria, other apoptotic proteins translocating to the cytosol of dying cells have been identified and have been shown to influence phenomena associated with apoptosis, such as apoptosis-inducing factor,²⁶ DIABLO/Smac^{3,5} and some mitochondrial caspases.^{27,28} To address the question whether tBid is able to induce the release of

such proteins, we used an *in vitro* reconstitution system in which isolated mouse liver mitochondria were treated with purified recombinant 15-kDa tBid. The tBid concentration used was such that no cytochrome *c* was released in the mitochondria from Bcl-2-transgenic mice. Subsequently, proteins released in a tBid-dependent way from mitochondria were identified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS).

Results

To address the question whether tBid is able to induce release of proteins other than cytochrome *c*, we set up a comparative experiment in which isolated liver mitochondria were exposed/unexposed to recombinant 15-kDa tBid. In order to use a relevant concentration of tBid, we determined the amount of tBid at which cytochrome *c* release is blocked by overexpression of Bcl-2. To that end, mitochondria of a Bcl-2 transgene, placed under control of the hepatocyte-specific α 1-antitrypsin gene promoter,²⁹ were exposed to a serial dilution of recombinant tBid. The release of cytochrome *c* induced by 6.7 nM (10 ng/100 μ l) of recombinant tBid was completely abrogated in liver mitochondria from Bcl-2-transgenic mice (Figure 1A). To determine whether the mitochondrial inner membrane was damaged, we checked for the presence of AK2 and AK3. AK2, an intermembrane mitochondrial protein, was shown to be released from mitochondria during the apoptotic process^{30,31} while AK3 is located exclusively in the mitochondrial matrix³² and resides there during the process of apoptosis.³³ As shown in Figure 1B, AK2 was specifically released with tBid, whereas AK3 could not be detected in the mitochondrial supernatant. This suggests that only intermembrane proteins had been released under the conditions used, that the inner mitochondrial membrane had not been damaged and that the matrix had remained intact.

To identify new mitochondrial proteins released with tBid, a large-scale preparation of isolated liver mitochondria

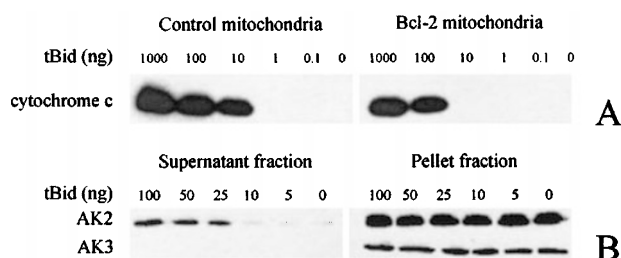


Figure 1 (A) tBid-induced cytochrome *c* release is blocked in Bcl-2-overexpressing mitochondria. Recombinant tBid was incubated with 40 μ g protein equivalent of liver mitochondria from control and liver-specific Bcl-2-transgenic mice. Supernatants were separated and subjected to 15% SDS-PAGE, followed by Western blotting using an anti-cytochrome *c* antibody. Ten ng of tBid (corresponding to 6.7 nM) was sufficient to trigger release of cytochrome *c* in control mitochondria, but no longer in Bcl-2 mitochondria. (B) tBid induces release of AK2, but not of AK3. tBid was incubated with a protein equivalent of 40 μ g purified mitochondria. Supernatants were separated from the mitochondrial pellets by centrifugation. The supernatant and pellet fractions were subjected to 15% SDS-PAGE, followed by immunoblotting with anti-AK2 antiserum. The same blots were stripped and analyzed for AK3 presence using an AK3-specific antibody

mitochondria was exposed, or not, to recombinant tBid. For purification purposes the resulting supernatant was separated on 15% SDS-PAGE gels (for low-molecular mass proteins) or 8% SDS-PAGE gels (for high-molecular mass proteins). Coomassie brilliant blue staining profiles revealed the presence of several tBid-inducible protein bands as compared to untreated controls (Figure 2). Differentially stained protein bands were excised from the gel containing the mitochondrial proteins released by tBid, together with the corresponding bands of the control lane. All excised proteins were *in-gel* digested using trypsin; a small fraction of the peptide mixture was used for MALDI-MS peptide mass fingerprinting. Since the proteins were separated only by their molecular mass, almost every excised protein band contained multiple proteins, which prevented unambiguous identification by peptide mass maps. Therefore the remainder of the peptide mixture was separated by reverse-phase HPLC, after which fractionated peptides were analyzed by MALDI-MS. First, all the peptides present in the protein band excised from the control lane were analyzed and used as a negative control. Next, the peptides from the tBid-dependent released protein band were analyzed (Figure 3A,B). Peptides that were solely present in the latter sample or were clearly dominant, were chosen for further sequence analysis by MALDI post-source decay (PSD) (Figure 3C). As an example, MALDI-PSD analysis of the protein sample identifying the serine protease Omi is shown (Figure 3).

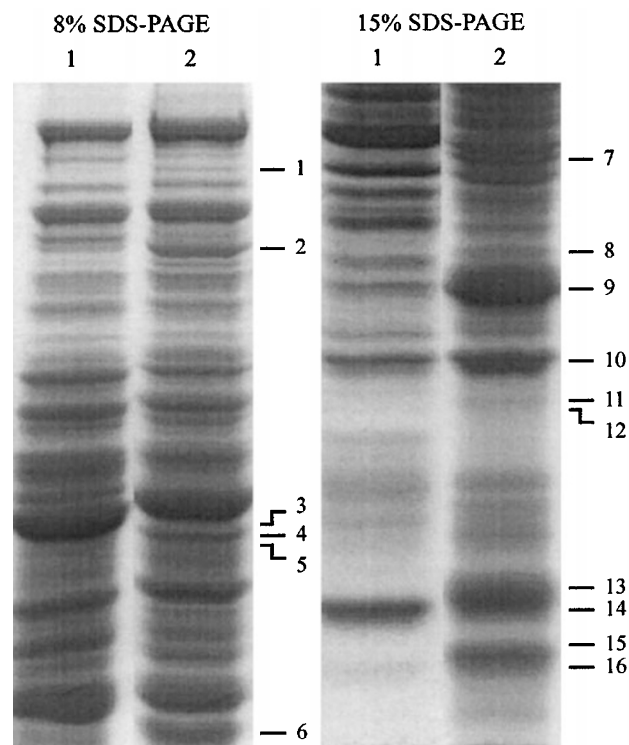


Figure 2 Coomassie brilliant blue staining profiles of tBid-induced mitochondrial supernatants isolated by centrifugation. Differential protein bands (separated by 8% and 15% SDS-PAGE) between controls (lanes 1) and tBid-induced supernatants (lanes 2) were excised and sequenced

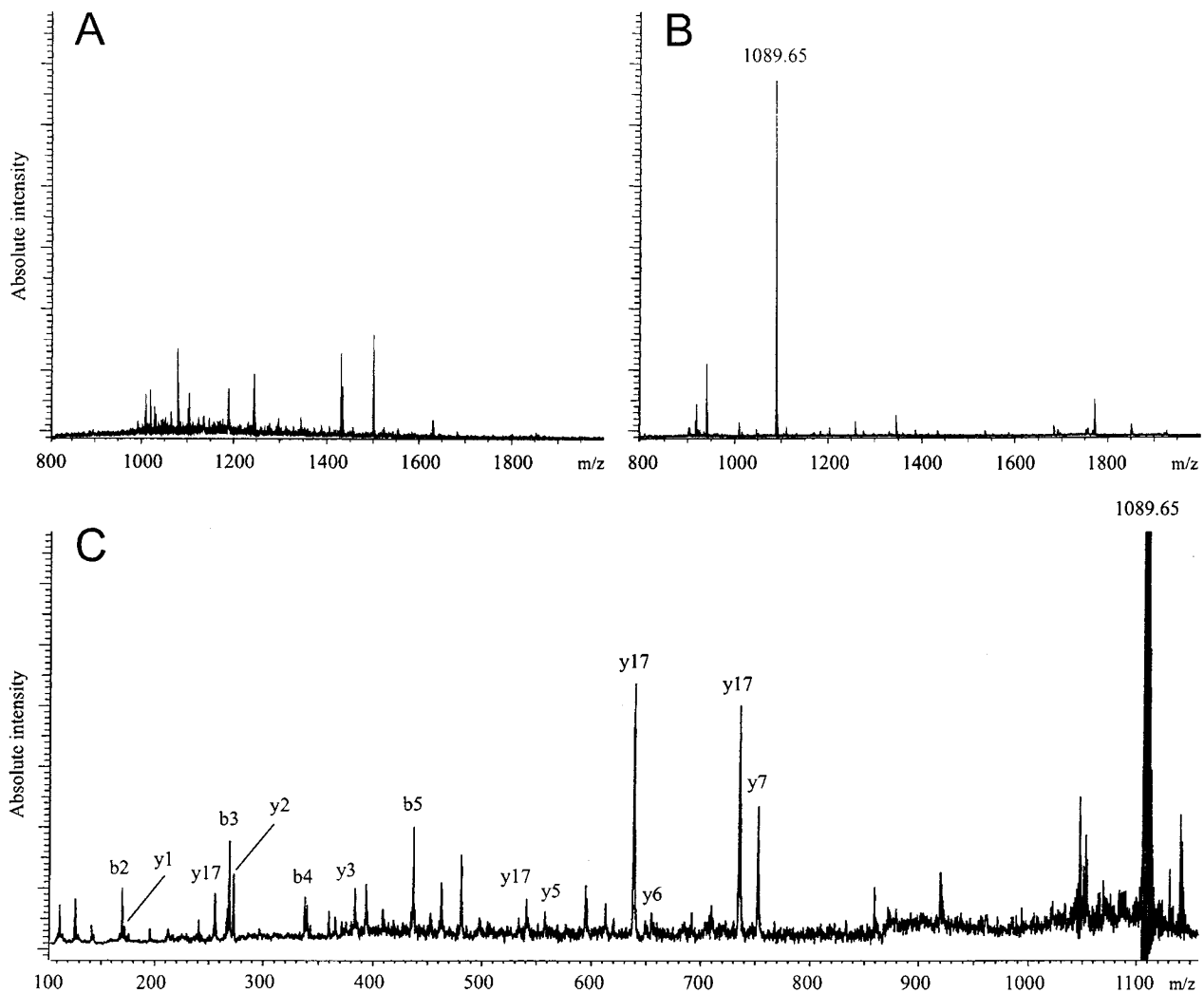


Figure 3 MALDI-MS identification of the Omi protein released by tBid. Protein band 6 and its control (Figure 2) were digested using trypsin. The peptide mixture generated was separated by reverse phase-HPLC. Shown are MALDI-MS spectra of peptides present in the HPLC fraction from protein digests in negative control (A) and tBid-released (B) proteins. (C) MALDI-PSD analysis of the peptide with a mass of 1089.65 Da present in the spectrum of B permitted to identify the sequence NH₂-AVPAPPPTSPR-COOH of the mouse Omi protein. This was further verified by manually checking the PSD spectrum for the presence of other tryptic peptide fragments, cumulatively covering approximately 23% of the amino acid sequence of the Omi protein (data not shown)

Using this approach, we identified proteins that were released in a recombinant tBid-dependent way and which were not present in the supernatant of untreated mitochondria (Table 1). They include proven apoptogenic proteins such as cytochrome *c* and DIABLO/Smac.^{3,5} We also detected AK2, a protein without clear function in apoptosis,^{30,31} and a number of proteins from a list of mitochondrial factors released by atractyloside *in vitro*, such as glutamate dehydrogenase, methylmalonate semi-aldehyde dehydrogenase, coproporphyrinogen III oxidase, fatty acid-binding protein and acyl-CoA-binding protein.³⁴ The functional impact of these proteins on apoptosis is unknown.

Identified was also endonuclease G, previously suggested to play a role in mitochondrial DNA replication,^{35,36} and recently found to be released from the mitochondria during apoptosis, where it might be involved in caspase-

independent DNA degradation.^{37–39} Endonuclease G seems to be one of the proteins most abundantly released, as can be appreciated from the 15% Coomassie brilliant blue gel (Table 1 and Figure 2).

Besides these known proteins some new factors were identified: a type-I RNA helicase, polypyrimidine tract-binding protein, the serine protease Omi, a WD-40 repeat-containing protein, actin-related protein 2/3 complex subunit 4 and translocase of inner mitochondrial membrane TIMM13b. Endoplasmic reticulum chaperone (also called GRP94), the major glycoprotein of the endoplasmic reticulum (ER), was identified as a tBid-released protein, probably due to a minor contamination of the mitochondrial cell fraction with the ER. This observation suggests that tBid has, besides its major activity on mitochondria, an additional effect on the ER, or that endoplasmic reticulum chaperone has a dual localization in the ER and the mitochondria.

Table 1 MALDI-PSD analysis and identification of mitochondrial proteins released by tBid

Band	Theor. m	Description	Access. No.	Coverage	PSD on peptide	Localization
1	123 kDa	Type-I RNA helicase Uptf1	gi 1885356	24%	GNTSGHIVNHLVR - IPSEQEQLR	CP, nucleus, MIT*
2	93 kDa	Endoplasmic	gi 119362	26%	NKEIFLR	ER
3	61 kDa	Glutamate dehydrogenase	gi 90522	34%	SEAAADREDDPNFFK - TAAVYVNAIEK	MIT (MAT)
4	57 kDa	Methylmalonate semialdehyde dehydrogenase	gi 400269	12%	SSFRGDTNFYK	MIT (MAT)
5	56 kDa	Polypyrimidine tract-binding protein	gi 2500586	23%	IAPGLAGAGNSVLLSNLPER	nucleus, MIT*
6	49 kDa	Serine protease Omi	gi 5739487	23%	AVPAPPPTSPR	MIT, ER
7	40 kDa	Coproporphyrinogen III oxidase	gi 547616	49%	N.D.	MIT (IMS)
8	32 kDa	WD-40 repeat tumor-specific antigen	gi 3123027	8%	KEELLNAMoxVAK	?
9	30 kDa	Endonuclease G	gi 6679647	28%	ASGLLFPNILAR	MIT (MAT (IMS?))
10	26 kDa	Adenylate kinase 2	gi 266401	19%	NGFLLDGFDR	MIT (IMS)
11	26 kDa	Smac/DIABLO	gi 8953909	29%	AVYTLVSLYR	MIT (IMS)
12	20 kDa	Aip2/3 complex subunit 4 (subunit p20-Arc)	gi 5031595	19%	AENFFILR	CP, MIT*
13	14 kDa	Fatty acid-binding protein	gi 6015126	63%	IKLTIYGPK	MIT, CP
14	14 kDa	Cytochrome c	gi 118008	53%	N.D.	MIT (IMS)
15	10 kDa	Translocase of inner mitochondrial membrane (TIMM13b)	gi 11024700	45%	VQIAVANAQELLQR	MIT (IMS)
16	10 kDa	Acyl-CoA-binding protein (diazepam-binding inhibitor)	gi 398990	41%	N.D.	MIT (MOM)

*The mitochondrial localization, still not described, is based on computational models (CP, cytoplasm; IMS, intermembrane space; MAT, matrix; MIT, mitochondria; MOM, mitochondrial outer membrane)

In most cases the difference between tBid-treated and untreated mitochondria led to the identification, in qualitative terms, of extra proteins in the treated condition. However, some protein bands showed a stronger staining in the control sample as compared to the tBid condition (Figure 2). Also in this case, we excised the band of similar height in the lane of tBid-treated mitochondria. The protein identification procedure used in this study is not able to document quantitative differences between protein mixtures. To this end, differential techniques that use stable isotope incorporation into peptides should have been employed.^{40,41} Nevertheless, MALDI-MS analysis of these protein bands did not reveal qualitative differences for these more intensely stained proteins in the case of supernatant from untreated mitochondria. Hence it is possible that a tBid-dependent release of proteases (such as Omi) partially degrades proteins in the mitochondrial supernatant, resulting in a lesser protein content.

Discussion

Although cytochrome *c* is the best known and best documented mitochondrial protein released during apoptosis, the mitochondria still contain other apoptogenic factors, such as DIABLO/Smac,^{3,5} apoptosis-inducing factor^{26,42} and mitochondrial caspases.^{27,28} To address the question whether tBid is able to induce the release of such proteins, we set up a comparative experiment in which isolated liver mitochondria were subjected to a relevant concentration of tBid, *viz* a concentration of tBid at which cytochrome *c* release is blocked by overexpression of Bcl-2. A proteome analysis was performed on the supernatant of isolated mitochondria (either untreated or treated with recombinant tBid) to determine any tBid-dependent release of proteins. Except for apoptosis-inducing factor and mitochondrial caspases, most mitochondrial proteins already described as being released during apoptosis were identified by our approach. Among the proteins released from the mitochondria during the apoptotic process we identified cytochrome *c*, DIABLO/Smac and AK2, as well as some proteins previously reported to be present in the supernatant of atractyloside-treated isolated mitochondria. The latter include glutamate dehydrogenase, methylmalonate semialdehyde dehydrogenase, coproporphyrinogen III oxidase, fatty acid-binding protein and acyl-CoA-binding protein.³⁴ Glutamate dehydrogenase and coproporphyrinogen III oxidase are implicated in oxidative deamination and heme biosynthesis, respectively. Methylmalonate semialdehyde dehydrogenase is involved in breakdown of branched amino acids. Fatty acid-binding protein binds free fatty acids and may be implicated in intracellular lipid transport. Acyl-CoA-binding protein, the endogenous ligand of the mitochondrial benzodiazepine receptor, is involved in acetyl-CoA ester transport and was reported to cause opening of the permeability transition pore after ligation with its receptor, thus favoring apoptosis.⁴³⁻⁴⁵ Furthermore, we identified the translocase of the inner mitochondrial membrane TIMM13b, a protein implicated in protein import from the cytoplasm to the mitochondrial inner membrane. Another member of this family, the X-linked deafness-dystonia protein (also called TIMM8a),⁴⁶ has already been described as being

released from the intermembrane mitochondrial space after treatment with atractyloside.³⁴ Also the recently published proapoptotic DNase endonuclease G, probably involved in caspase-independent DNA degradation, was clearly identified.^{37–39}

We also identified a few new proteins released from tBid-treated mitochondria, some of which may modulate apoptotic pathways. An interesting finding is tBid-mediated release of Omi (also named HtrA2), a serine protease homologous to the bacterial heat shock endoprotease HtrA. Omi contains a mitochondrial localization signal⁴⁷ and was suggested to play a role in the degradation of aberrantly folded proteins during cellular stress caused by reactive oxygen species, toxins or ischemia/reperfusion.^{48,49} As tBid induces release of Omi from mitochondria, it is conceivable that its protease activity, once translocated to the cytosol, may regulate apoptotic proteolytic cascades or may proteolyze some substrates implicated in apoptosis. Although caspases, a family of cysteine proteases, are the main players in the apoptotic process, other non-caspase proteases have been described as being involved in apoptosis, including cathepsins, calpains, serine proteases and the proteasome complex.⁵⁰

Another interesting tBid-released protein that may modulate apoptotic pathways, is the polypyrimidine tract-binding (PTB) protein. Little is known about the precise function of this protein, but it was recently recognized as an important player in alternative splicing regulation.⁵¹ In the context of apoptosis, the PTB protein was shown to bind downstream of the alternative exon 9 of caspase-2, thus preventing inclusion of the alternative exon 9.⁵² Caspase-2L is derived from the skipping of alternative exon 9 and can induce cell death in a variety of cells,^{53,54} whereas caspase-2S is a truncated version of the protein due to inclusion of exon 9 (containing a premature termination codon). Since overexpression of caspase-2S prevents apoptotic cell death,^{53,55} release of the PTB protein might favor formation of the proapoptotic caspase-2L form. Most likely, the PTB protein is also involved in the splice regulation of other, possibly apoptosis-related, proteins.

A third potentially interesting protein identified is a WD-40 repeat-containing protein. Although the function of the protein is essentially unknown,⁵⁶ WD-40 repeats are present in the C-terminal regulatory domain of Apaf-1. They were shown to be involved in self-association of Apaf-1 and regulation of caspase-9 activation.^{57–59} WD-40 repeats are present in a variety of proteins with diverse functions and have been proposed to play a regulatory role.⁶⁰

Also Arp2/3 complex subunit 4 (known as p20-Arc) was identified. The Arp2/3 protein complex is implicated in the control of actin polymerization. The complex consists of seven subunits including the actin-related proteins Arp2 and Arp3, and five others referred to as p41-Arc, p34-Arc, p21-Arc, p20-Arc and p16-Arc.⁶¹ Recent observations support a role for the Arp2/3 complex and/or actin polymerization in the movement of organelles in eukaryotic cells and yeast.^{62,63} Although the precise localization of the different components is unknown, Arp2/3 complex subunits colocalize with the mitochondria in intact yeast.⁶³ Thus it is possible that the Arp2/3 complex subunit 4 identified here

plays a role in cytoskeletal rearrangements and/or mitochondrial changes in the ultrastructural organization during the process of apoptosis.⁶⁴

Finally, we also found the type-I RNA helicase Upf1 as being released under influence of tBid. Upf1 is involved in regulation of translation by operating in the nonsense-mediated decay of mRNA.^{65,66} It is remarkable that our protein analysis revealed two large proteins, *viz.* 123-kDa type-I RNA helicase and 93-kDa endoplasmic reticulum chaperone. In fact, the maximum protein size for mitochondrially released proteins is considered to be approximately ~80 kDa.³⁴ However, it is not clear if this release is associated with an action of tBid on the mitochondria, since Upf1 is associated with polyribosomes or resides in the cytosol, depending on its phosphorylation status.⁶⁷ Endoplasmic reticulum chaperone (also called GRP94), a hsp90 member of glucose-regulated proteins, was described as a chaperone associated with the ER.⁶⁸ Endoplasmic reticulum chaperone has been reported to have antiapoptotic properties and to be a proteolytic target for calpains during etoposide-induced apoptosis.⁶⁹ Although a mitochondria-associated localization of these two proteins cannot be excluded, their presence in the supernatant of tBid-treated mitochondria may reflect a contamination of the isolated mitochondria with the ER; this may also imply that tBid has targets other than mitochondria. In this respect, Bcl-2 was also shown to localize to the ER.^{70,71}

As expected, most of the proteins identified have been reported to have a mitochondrial subcellular localization. Moreover, Western blotting revealed that the release of proteins is most probably confined to the intermembrane space, since AK2 (but not the soluble matrix protein AK3) was shown to be present in the supernatant of tBid-treated mitochondria. Although glutamate dehydrogenase and methylmalonate semialdehyde dehydrogenase are matrix proteins, part of the proteins may be localized in the intermembrane space of the mitochondria. The same might be true for endonuclease G, which plays a role in mitochondrial DNA replication³⁶, favoring the idea of matrix localization. Alternatively, one cannot exclude the possibility that matrix proteins are released from the mitochondria under certain conditions.

Our *in vitro* study clearly shows that a proteome analysis by MALDI-MS and PSD is a powerful tool to study the release of mitochondrial proteins under conditions of apoptotic signaling. Besides known apoptogenic mitochondrial proteins, some new potentially apoptogenic proteins were identified. Further investigation into the involvement of these proteins in the apoptotic process will permit to understand the function of their mitochondrial release, also under other pathophysiological conditions in which mitochondrial dysfunction occurs, such as senescence.

Materials and Methods

Isolation of murine liver mitochondria

C57BL/6 wild-type and Bcl-2-transgenic mice (kindly provided by Dr. I Rodriguez, Laboratory of Vertebrate Neurobiology, The Rockefeller University, New York, NY, USA)²⁹ were used at the age of 7–12

weeks. Transgenic offspring were identified by PCR using Bcl-2-specific primers. Livers of wild-type and Bcl-2-transgenic mice were homogenized in homogenization buffer (5 mM KH_2PO_4 pH 7.4, 0.3 M sucrose, 1 mM EGTA, 5 mM 3-(N-morpholino)propanesulfonic acid). Mitochondria were purified as described previously^{39,72} and resuspended in cell-free system buffer (10 mM HEPES-NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4 , 0.5 mM EGTA, 2 mM MgCl_2 , 5 mM pyruvate, 0.1 mM PMSF, and 1 mM dithiothreitol), kept on ice and used within 1 h of preparation.

Analysis of tBid-induced release of mitochondrial proteins

Mitochondria equivalent to 40 μg of liver protein were incubated at 37°C in 100 μl cell-free system buffer for 20 min with a serial dilution of recombinant tBid. Supernatants were separated from mitochondria by centrifugation at 20 000 $\times g$ for 10 min at 4°C. 1/5 of supernatant was subjected to 15% SDS-PAGE, followed by Western blotting. Antibodies used for Western blots were anti-cytochrome *c* (clone 7H8.2C12; PharMingen, San Diego, CA, USA), anti-AK2 and anti-AK3 (kindly provided by Dr. T Noma, Department of Biochemistry, Yamaguchi University School of Medicine, Yamaguchi, Japan). Blots were visualized with the chemiluminescence NEN Renaissance method (Du Pont, Wilmington, DE, USA) after incubation of membranes with secondary antibodies coupled to horseradish peroxidase (Amersham Pharmacia Biotech, UK).

Protein and peptide preparation procedure

In order to identify released proteins by MS, a mitochondrial equivalent of one liver was prepared (corresponding to approximately 1 mg protein) and incubated with 170 nM purified tBid for 20 min at 37°C (500 ng tBid/200 μl of mitochondria equivalent to 1000 μg of liver protein). Supernatant was prepared by 20 000 $\times g$ centrifugation for 10 min at 4°C and submitted to 15% SDS-PAGE analysis. Coomassie brilliant blue-stained bands corresponding to tBid-induced mitochondrial protein release were excised and transferred to individually wrapped Eppendorf tubes. Corresponding gel slices of a parallel run lane of proteins spontaneously released or dissociated from unstimulated mitochondria were used as negative controls. Gel slices were digested using trypsin, as described previously.⁷³ After digestion, supernatant containing tryptic peptides was removed from the gel pieces and acidified using 1 μl formic acid. A small fraction (10%) of this mixture was concentrated on Poros 50 R2 beads (Roche Molecular Biochemicals, Basel, Switzerland) and used for MALDI-MS peptide mass-fingerprint analysis as previously described.⁷⁴ Since the excised gel bands contained multiple proteins, no unambiguous protein identification was possible by solely using the obtained tryptic peptide mass maps. Therefore the remainder of the peptide mixture was loaded on a 1 \times 50 mm Vydac C18-column (The Separations Group, Hesperia, CA, USA); peptides were separated by reverse phase-HPLC using an acetonitrile gradient. Eluting peptides were automatically collected in an aqueous solution containing a small number of Poros 50 R2 beads.⁷³ These fractions were completely dried in a centrifugal vacuum concentrator and stored at -20°C for further analysis by MALDI-MS.

MALDI-MS protein identification

All MALDI-MS experiments were performed on a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The peptides present in each reverse phase-HPLC fraction

were first scanned in reflectron mode; peptides that were clearly enriched as compared to the negative control sample were further selected for MALDI-PSD analysis. The peptide fragmentation spectra obtained were automatically used for protein identification in a nonredundant protein database using MASCOT algorithm (<http://www.matrixscience.com>).

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