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Mass spectrometric identification of proteins released from mitochondria undergoing permeability transition

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Abstract

Mitochondrial membrane permeabilization is a rate-limiting step of cell death. This process is, at least in part, mediated by opening of the permeability transition pore complex (PTPC) Several soluble proteins from the mitochondrial intermembrane space and matrix are involved in the activation of catabolic hydrolases including caspases and nucleases. We therefore investigated the composition of a mixture of proteins released from purified mitochondria upon PTPC opening. This mixture was subjected to a novel proteomics/mass spectrometric approach designed to identify a maximum of peptides. Peptides from a total of 79 known proteins or genes were identified. In addition, 21 matches with expressed sequence tags (EST) were obtained. Among the known proteins, several may have indirect or direct pro-apoptotic properties. Thus endozepine, a ligand of the peripheral benzodiazepin receptor (whose occupation may facilitate mitochondrial membrane permeabilization), was found among the released proteins. Several proteins involved in protein import were also released, namely the so-called X-linked deafness dystonia protein (DDP) and the glucose regulated protein 75 (grb75), meaning that protein import may become irreversibly disrupted in mitochondria of apoptotic cells. In addition, a number of catabolic enzymes are detected: arginase 1 (which degrades arginine), sulfite oxidase (which degrades sulfur amino acids), and epoxide hydrolase. Although the functional impact of each of these proteins on apoptosis remains elusive, the present data bank of mitochondrial proteins released upon PTPC opening should help further elucidation of the death process. *Cell Death and Differentiation* (2000) **7**, 137–144.

Keywords: apoptosis; cytochrome c; mitochondria; permeability transition; mass spectrometry; proteomics

Abbreviations: AIF, apoptosis inducing factor; ANT, adenine nucleotide translocator; DDP, X-linked deafness dystonia protein; MS, mass spectrometry; PTPC, permeability transition pore complex; SIMPs, soluble intermembrane space proteins; VDAC, voltage dependent anion channel

Introduction

Apoptosis is (almost) uniformly accompanied by an increase in mitochondrial membrane permeability,[1,2] that is triggered by an ever increasing number of different pro-apoptotic effectors, including caspases,[3,4] pro-apoptotic members of the Bcl-2/Bax family,[5–7] lipid second messengers such as ganglioside GD3,[8] Ca^{2+}, or pro-oxidants.[9–11] The permeabilization of the inner membrane is partial, allowing for the free diffusion of solutes of up to 1500 Da and may be reversible.[1,2] In contrast, permeabilization of the outer membrane is complete, culminating in the release of soluble intermembrane space proteins (SIMPs). The mechanism accounting for membrane permeabilization is a matter of debate. At least in some models, it involves proteins of the permeability transition pore complex (PTPC) whose most abundant components, the voltage dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT), interact with pro- and anti-apoptotic members of the Bcl-2/Bax family.[6,12] How SIMPs are released from mitochondria is not understood. According to one hypothesis, the liberation of SIMPs may result from ANT-mediated inner membrane permeabilization[6,13] with consequent matrix swelling and physical disruption of the outer membrane.[14] However, it has also been suggested that VDAC may form a protein-permeable conduit.[12]

Irrespective of the exact mode of outer membrane permeabilization, it appears that the release of SIMPs occurs in a non-selective fashion and indistinguishably affects relatively small proteins such as cytochrome *c* (14.5 kDa),[15] as well as larger proteins such as the adenylate kinase-2 (50 kDa),[16,17] apoptosis inducing factor (AIF, 57 kDa),[18,19] and mitochondrial caspases (up to 50 kDa).[20,21] Several among these SIMPs are highly apoptogenic, thus providing a molecular link between mitochondrial dysfunction and the activation of catabolic hydrolases. Thus, cytochrome *c* and hsp 10 (which translocate into the cytosol) participate in the activation of caspases within the apoptosome,[15,22] whereas AIF (which translocates into the nucleus) activates endonucleases and may account for caspase-independent large scale chromatin condensation.[19,23]

Intrigued by the fact that several mitochondrial proteins are involved in the apoptotic process, we decided to investigate the composition of a mixture of proteins released from purified mitochondria upon PTPC opening.

This mixture was subjected to a novel mass spectrometric approach designed to identify as many peptides as possible.[24–26] Here we report the identification of 97 mitochondrial proteins, some of which may contribute to the cell death cascade.

Results and Discussion

Creation of a protein data base

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was employed to investigate the peptide composition of trypsin-digested supernatants, obtained from mouse liver mitochondria treated with the PTPC-opening agent atractyloside. This supernatant contained multiple proteins with a maximum size of ~80 kDa (Figure 1) and clearly differed in its composition from other submitochondrial fractions. Thus, when compared to the intermembrane space fraction of proteins, a lower content of proteins with a molecular mass greater than 80 kDa and a clear bias in favor of smaller proteins was found (Figure 1). The LC-MS/MS technology was applied either to the unfractionated peptide mixture or to a batch of peptides that had been enriched based on their cysteine content, as detailed in Materials and Methods. Proteins were identified by correlation of their peptide sequence with an uninterpreted MS/MS spectrum. Obvious contaminants (trypsin and 15 peptides corresponding to 10 different ribosomal proteins) were excluded from the pool of proteins which are listed in Table 1. Peptides corresponding to a total of 79 known proteins or genes were identified. In addition, 21 matches with expressed sequence tags (EST) were obtained. Of note, several SIMPs that are known to be released from mouse liver mitochondria exposed to atractyloside (such as AIF or caspases 2 and 9)[19,20] were not detected, indicating that this protein data base must be regarded as incomplete. These proteins are most likely at levels at or below that of the contaminants (ribosomal proteins) identified in the sample.

As to be expected, most of the proteins whose subcellular localization has been previously assessed are mitochondrial proteins. A lysosomal contamination might be suggested by the detection of cathepsin B.

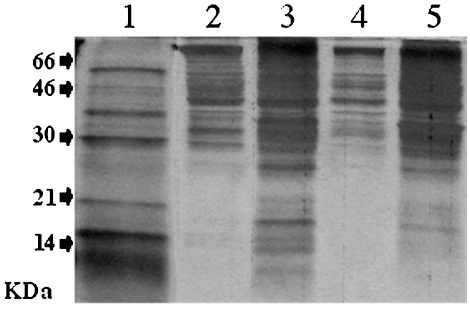

Figure 1 Silver staining profiles of the supernatant of atractyloside treated mouse liver mitochondria as well as submitochondrial fractions. Lane 1: supernatant of atractyloside-treated mitochondria (5 mM, 30 min). Lane 2: Inner membrane proteins. Lane 3: intermembrane proteins. Lane 4: outer membrane. Lane 5: matrix

However, cathepsin B has been previously found in the supernatant of atractyloside treated mitochondria,[27] and this may indicate that some of this protease is actually imported into mitochondria and/or associates with mitochondria. Several proteins which are normally considered as peroxisomal were also detected. Again, it is possible that this is due to a minor contamination of mitochondria with peroxisomes. Alternatively, it may be indicative of a dual (peroxisomal plus mitochondrial) localization of such proteins, which has been observed.[28–30] A large number of proteins that are classified as matrix proteins were identified. This may constitute a fraction of proteins that are being imported (through the intermembrane space) or, alternatively, suggest that some mitochondria (partially?) lose the integrity of the inner mitochondrial membrane.[22,31] In accord with this latter possibility, mitochondria treated with the PTPC activator Bax release the matrix protein aspartate aminotransferase[32] and some translocation of the matrix protein hsp60 into the cytosol has been reported to occur in cells undergoing apoptosis.[22] The release of hsp60, however, is not detectable by immunofluorescence staining, indicating that it only affects a minor fraction of the pool of hsp60.[22,31] We have verified the subcellular localization of two proteins found in the data base, namely X-linked deafness dystonia protein (DDP, which is an intermembrane protein) and SOD2 (which is a matrix protein). As to be expected from their submitochondrial localization, DDP distributed from mitochondria (counterstained with the matrix protein hsp60) to the cytosol when apoptosis was induced (Figure 2A). In contrast, SOD2 remained mitochondrial (Figure 2B), meaning that only a minority of SOD2, if any, was liberated from mitochondria following apoptosis induction.

Concluding remarks

The data contained in Table 1 confirm the non-selective nature of mitochondrial membrane permeabilization. In addition to known apoptogenic SIMPs, a number of different proteins which might have some impact on cell death were identified. Thus acyl-CoA binding protein (diazepam binding inhibitor, also called endozepine) and a putative analogue (corresponding to EST AA930535) were found in the mitochondrial supernatant. We and others have found in the past that ligation of the mitochondrial benzodiazepine receptor (whose endogenous ligand is acyl-CoA binding protein) causes PT pore opening and favors apoptosis.[33–36] It is tempting to speculate that endozepine released from mitochondria undergoing PT may affect other mitochondria. Several proteins involved in protein import were also released, namely the so-called X-linked deafness dystonia protein (DDP)[37] and the glucose regulated protein 75 (grb75). This may imply that protein import becomes disrupted in mitochondria, making the outer membrane damage irreversible. Moreover, grb75 has been reported to interact with p53.[38] The mass spectrometric data indicate that several antioxidant enzymes were released from

Table 1 Mass spectrometric identification of mitochondrial proteins

Accession Code	Protein description	Species	Localization of mature protein	Peptide or No. of peptides*	Occurrences
Q64433	10 kd heat shock protein, mitochondrial (hsp10)	mouse	intermembrane	6	14
Q64591	2,4-dienoyl-CoA reductase, mitochondrial precursor (ec 1.3.1.34)	rat	mitoch.	2	2
P42125	3,2-trans-enoyl-CoA isomerase, mitochondrial precursor (ec 5.3.3.8)-	mouse	matrix	VLVETEG-PAGVAVMK	1
W64336	3-ketoacyl-CoA thiolase mitochondrial (ec 2.3.1.16)	mouse	matrix	12	21
P21775	3-ketoacyl-CoA thiolase peroxisomal a precursor (ec 2.3.1.16)	rat	peroxisome	6	13
P07871	3-ketoacyl-CoA thiolase peroxisomal b precursor (ec 2.3.1.16)	rat	matrix	15	25
P20029	78 kd glucose regulated protein precursor (grp 78)	mouse	matrix	2	2
P17764	acetyl-CoA acetyltransferase precursor, mitochondrial (ec 2.3.1.9)	rat	matrix	7	15
Q99798	aconitate hydratase, mitochondrial precursor (ec 4.2.1.3)	human	mitoch.	2	2
P51174	acyl-CoA dehydrogenase, long-chain specific precursor (ec 1.3.99.13)	mouse	mitoch.	CIGAIAM-TEPGAG-SDLQGVR	1
P31786	acyl-CoA-binding protein (diazepam binding inhibitor) (endozepine)	mouse	mitoch.	2	2
P07872	acyl-CoA oxidase, peroxisomal (ec 1.3.3.6)	rat	mitoch.	2	2
P54819	adenylate kinase isoenzyme 2, mitochondrial (ec 2.7.4.3)	human	intermembrane	5	7
P46656	adrenodoxin precursor (adrenal ferredoxin)	mouse	matrix	LGCQVCLTK	2
AF027730	alanine:glyoxylate aminotransferase	mouse	mitoch. peroxisomes	VIHHTTPVT-SLYCLR	1
P47738	aldehyde dehydrogenase, mitochondrial precursor (ec 1.2.1.3)	mouse	matrix	9	10
P49419	antiquitin (ec 1.2.1.-)	human		GSDCGIVNV-NIPTSGAEIG-GAFG	1
Q61176	arginase 1 (ec 3.5.3.1)	mouse	mitoch.	6	7
P16460	argininosuccinate synthase (ec 6.3.4.5)	mouse	cytosol	FELTCYSLAP-QIK	2
P05202	aspartate aminotransferase, mitochondrial precursor (ec 2.6.1.1)	mouse	matrix	7	8
Q06185	ATP synthase e chain, mitochondrial (ec 3.6.1.34)	mouse	inner membrane	ELAEAQDD-SILK	1
AF033381	betaine homocysteine methyl transferase	mouse	cytosol	7	7
P07756	carbamoyl-phosphate synthase (ammonia) mitochondrial precursor (ec 6.3.4.16)	rat	matrix	33	45
P24270	catalase (ec 1.11.1.6)	mouse	peroxisomes	24	48
P10605	cathepsin b precursor (ec 3.4.22.1) (cathepsin b1)	mouse	lysosomes	EQWSNCP-TIGQIR	1
U90725	caveolae-associated protein mRNA; high-density lipoprotein binding protein	rat	caveolae	LVGEIM-QETGTR	1
AF004591	copper transport protein Atox1 (ATOX1)	mouse		VCID-SEHSSDTL-LATLNK	1
P36552	coproporphyrinogen iii oxidase precursor (ec1.3.3.3.) (coproporphyrinogenase)	mouse	mitoch.	2	2
P00009	cytochrome c	mouse	intermembrane	6	11
Q01205	dihydrolipoamide succinyltransferase (ec 2.3.1.61)	rat	inner membrane maxrix	GLVVPVIR	1
L79910	dimethylglycine dehydrogenase like protein	rat	matrix	GAQVIEN-CAVTGIR	1
P13803	electron transfer flavoprotein alpha-subunit precursor	rat	inner membrane	15	29
P04720	elongation factor 1-alpha 2 (ef-1-alpha-2)	human	cytoskeleton, cytosol	IGGIGTVP-VGR	1
P30084	enoyl-CoA hydratase, mitochondrial precursor (ec 4.2.1.17)	human	mitoch.	3	3

(Continued)

Table 1 ***(Continued)***

Accession Code	Protein Description	Species	Localization of mature protein	Peptide of No. of peptides	Occurrences
AA472254	enoyl-CoA: hydratase-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (similar to GB:L07077)	mouse	mitoch. peroxisome	LGILDVVVK	1
P51660	estradiol 17 beta-dehydrogenase 4 (ec 1.1.1.62)	mouse		2	2
P12710	fatty acid-binding protein, liver (l-fabp) (14 kd selenium-binding protein)	mouse	mitoch. cytosol	4	6
P00884	fructose-bisphophate aldolase b (ec 4.1.2.13)	rat		5	9
P26443	glutamate dehydrogenase precursor (ec 1.4.1.3)	mouse	matrix	6	8
P11352	glutathione peroxidase (ec 1.11.1.9) (gshpx-1)	mouse	mitoch.,cytosol peroxisome	2	2
P23434	glycine cleavage system h protein precursor	human	mitoch.	SCYEDGWLIK	1
P29411	gtp:amp phosphotransferase mitochondrial (ec 2.7.4.10)	rat	mitoch.	AYEAQ-TEPVLQYYQK	1
P34931	heat shock 70 kd protein 1-hom (hsp70 hom)	human		TTPSYVAFTD-TER	2
P52760	heat-responsive protein 12	mouse		APAAIGPYS-QAVQVDR	2
Q00341	high density lipiprotein binding protein (hdl-binding protein)	human		2	3
P38060	hydroxymethylglutaryl-CoA lyase precursor (ec 4.1.3.4)	mouse	mitoch. peroxisomes	EVSVFGAV-SELFTR	1
P54869	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor (4.1.3.5)	mouse	mitoch.	2	2
P12007	isovaleryl-CoA dehydrogenase precursor (ec 1.3.99.10)	rat	matrix	2	3
M89644	keratin K5	rat	cytoskeleton	SLDLDSII-AEVK	1
A61281	lysozyme homolog AT 2, bone	rat		NTDGSTDY-GILQINSR	1
W11382	ma86g01.r1 Soares mouse p3NMF19. W11382 Frame +3	mouse		VTHLSTLQV-GSLSVK	1
P08249	malate dehydrogenase, mitochondrial precursor [acylating] (ec 1.2.1.27)	mouse	matrix	12	16
Q02253	methylmalonate-semialdehyde dehydrogenase precursor [acylating] (ec 1.2.1.27)	rat	mitoch.	3	3
P19226	mitochondrial matrix protein p1 precursor (p60 lymphocyte protein)	mouse	matrix	16	27
P38646	mitochondrial stress-70 protein precursor (75 kd glucose regulated protein)	human	mitoch.	7	10
P20108	mitochondrial thioredoxin-dependent peroxide reductase precursor	mouse	mitoch.	GLFIIDPN-GVVK	1
P32020	nonspecific lipid-transfer protein precursor (sterol carrier protein 2)	mouse	mitoch. peroxisome	15	45
P15531	nucleoside diphosphate kinase a (ec 2.7.4.6)	human	matrix nuclear	4	5
P11725	ornithine carbamolyltransferase precursor (ec 2.1.3.3)	mouse	mitoch.	4	4
P05092	peptidyl-prolyl cis-trans isomerase a (ec 5.2.1.8) (cycophilin A)	human	cytosol	HTGPGILSMA-NAGPNTNGS-QFFICTAK	1
AF006688	peroxisomal acyl-CoA oxidase	mouse	peroxisome	2	2
P29341	polyadenylate-binding protein 1 (poly(a) binding protein 1)	mouse	cytosol	6	7
P09103	protein disulfide isomerase precursor (pdi) (ec 5.3.4.1) (prolyl 4-hydroxylase	mouse	ER	VDATEES-DLAQQYGVR	1
P99029	putative peroxisomal antioxidant enzyme (liver tissue 2d-page spot 2d-0014iv)	mouse	peroxisome	2	2
Q05920	pyruvate carboxylase precursor (ec 6.4.1.1)	mouse	matrix	2	2
P09139	serine-pyruvate aminotransferase, mitochondrial percursor (ec 2.6.1.51)	rat	mitoch	LLLGPGSN-LAPR	1
P34914	soluble epoxide hydrolase (ec 3.3.2.3) (epoxide hydratase)	mouse	cytosol, perox. mitoch.	2	2

(Continued)

Table 1 ***(Continued)***

Accession Code	Protein Description	Species	Localization of mature protein	Peptide of No. of peptides	Occurrences
P13086	succinyl-CoA ligase, alpha-chain precursor (ec 6.2.1.4)	rat	mitoch.	2	3
Q07116	sulfite oxidase precursor (ec 1.8.3.1)	rat	intermembrane	LCDVLA-QAGHR	2
P07895	superoxide dismutase [mn] precursor (ec 1.15.1.1)	rat	matrix	GDVTTQVAL-QPALK	1
P10639	thioredoxin	mouse		2	2
Q06830	thioredoxin peroxidase 2	human		4	10
P52196	thiosulfate sulfurtransferase (ec 2.8.1.1) (rhodanese)	mouse	matrix	5	6
P25688	uricase (ec 1.7.3.3)	mouse	peroxisomes	2	2
AF086630	VAMP-associated protein A	rat		3	3
U66035	X-linked deafness dystonia protein (DDP)	human	intermembrane	4	6
AI663509	uk33g02.y1, Sugano mouse kidney mkia Frame+2	mouse		MSGGSLIPSP-NQQALSPQP-SR	1
AF066751	ES/130 mRNA, complete cds; similar to 180K ribosome receptor and cardiac morphogenesis protein ES/130	human		3	4
H35434	EST105154 Rat PC-12 cells, untre H35434 Frame +3	rat		AGIVQDEVQ-PPGLK	2
W08097	mb39c09.r1 Soares mouse p3NMF19. W08097 Frame +1	mouse		2	2
W07953	mb45g02.r1 Soares mouse p3NMF19. W07953 Frame +2	mouse		AVATLQ-GEGLSVT-GIVCHVGK	1
W29591	mc08a08.r1 Soares mouse p3NMF19. W29591 Frame +1	mouse		RDPVDTDDT-ATALR	2
W62507	md72e10.r1 Soares mouse embryo N W62507 Frame +1	mouse		GTPEQPCGF-SNAVVQILR	1
W66688	me26a03.r1 Soares mouse embryo N W66688 Frame +1	mouse		3	6
W97106	mf90a04.r1 Soares mouse embryo N W97106 Frame +3	mouse		ASQQDFE-NALNQVK	1
AA008039	mg71f09.r1 Soares mouse embryo AA008039 Frame +2	mouse		FDSNVSGQS-SFGTSPAAD-NIEK	2
AA004027	mg80g01.r1 Soares mouse embryo AA004027 Frame +2	mouse		VQIAVANA-QELLQR	1
AA109583	ml97h06.r1 Stratagene mouse ki AA109583 Frame +3	mouse		FLYTVPNGN-NPTGNS-LTGDR	1
AA109917	mp49h11.r1 Barstead MPLRB1 Mus AA109917 Frame +3	mouse		NHLPVPNLD-PHTYR	1
AA222445	my 18g05.r1 Barstead mouse hear AA222445 Frame +2	mouse		IPNQFQGSP-PTPSDESVK	2
AA254902	mz78f08.r1 Soares mouse NML My AA254902 Frame +1	mouse		NQEAMGAF-QEFPQ-VEACR	1
AA939535	vsbob01.r1, similar to SW: ENDR_BOVIN POT106 Endozepine related protein precursor	mouse		2	2
W42142	similar to PIR:S16967 NADH dehydrogenase	mouse		2	2
W54192	similar to SW: YA94_SCHPO Q09783 Hypothetical 11.4 kD protein	mouse		CIGKPGGSL-DNSEQK	2
AA261575	similar to WP:B0334.3A CE02934 Oxalyl0CoA decarboxylase	mouse		TPEELQHSLR	1
AA002289	similar to WP:C16C10.11 CE01491	mouse		QFLECAQNQ-SDVK	2
H20248	yn56g04.r1 Homo sapiens cDNA clo H20248 Frame +1	human		LLADPT-GAFGK	1
TOTALS:	**Total # proteins/genes= 97** **# Known proteins/genes= 76** **# EST matches= 21**			298	255

*Peptide sequences are listed when only one set of mass spectroscopic data matched the corresponding data bank entry. In the case of more than one match, their number is specified

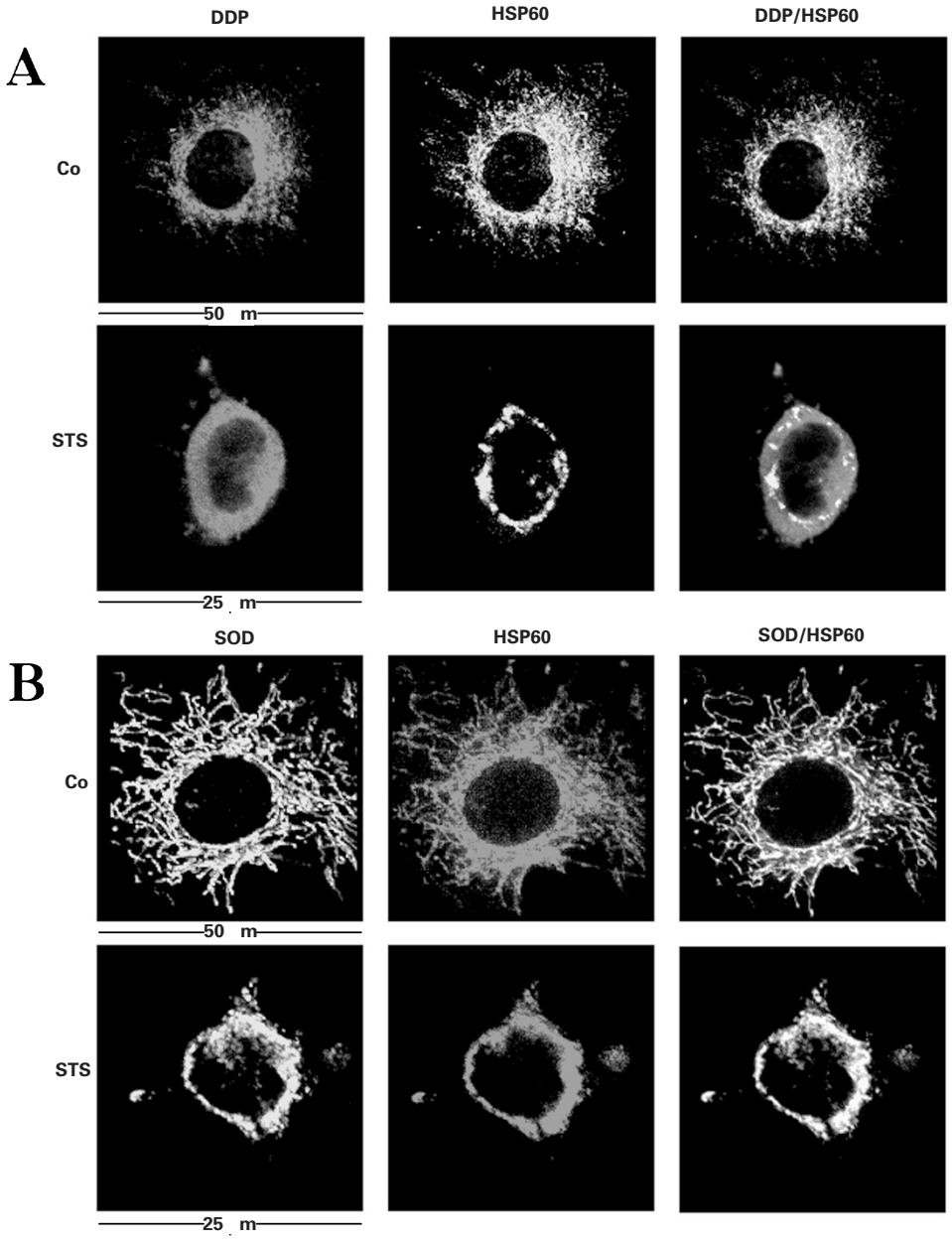

Figure 2 Subcellular distribution of DDP and SOD2 in cells undergoing apoptosis. Cells cultured in the absence (Co.) or presence of the apoptosis inducer STS were stained with antibodies specific for DDP (**A**) or SOD2 (**B**) and counterstained for the mitochondral matrix protein Hsp60. Note that the overlap between DDP and Hsp60 dependent fluorescence found in normal cells is largely abolished in apoptotic cells. In contrast, this overlap persists for SOD2 and Hsp60, even after induction of apoptosis

mitochondria. This applies to glutathione peroxidase, thioredoxin, thioredoxin peroxidase, and perhaps a fraction of manganese-dependent superoxide dismutase (SOD2), suggesting a relative deprotection of mitochondrial membranes from oxidative reactions. Accordingly, an enhanced local generation of superoxide anion and the oxidation of mitochondrial cardiolipin has been found to occur shortly after mitochondrial membrane permeabilization in intact cells.[39] Finally, a number of catabolic enzymes are detected in the SIMPs fraction: arginase 1 (which degrades arginine), sulfite oxidase (which degrades sulfur amino acids), and epoxide hydrolase. Such enzymes might deplete essential metabolites, thereby decreasing anabolic reactions and/or affecting energy metabolism.

The evaluation of each of these proteins in the context of apoptosis remains elusive. However, the data base of mitochondrial proteins contained in this article should prove useful for further investigation of cell death mechanisms.

Materials and Methods

Preparation of protein samples

Mitochondria (1 mg protein/ml) were purified from mouse liver on a Percoll gradient as previously described,[40] resuspended in CFS buffer (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 phenylmethyl sulfonylfluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml antipain, 10 μg/ml chymopapain, 1 mM dithiotreitol, 10 mM HEPES-NaOH, pH 7.4) and incubated in the presence of atractyloside (5 mM, 30 min, RT), followed by centrifugation ($7000 \times g$, 10 min, 4°C), recovery of the supernatant and ultracentrifugation ($1.5 \times 10^5 \times g$, 1 h, 4°C). This sample was subjected to LC-MS/MS. In addition, submitochondrial fractions were purified by standard procedures[41] and the samples were analyzed by SDS–PAGE (15%), followed by silver staining.

LC-MS/MS of unfractionated proteins

Forty μg of protein was dried, resuspended in 500 μL of 30 mM Tris-HCl, pH 7.0/8 M urea/10 mM dithiotreitol, and incubated for 30 min at 37°C. The sample was alkylated with 20 mM iodoacetamide for 30 min at room temperature in the dark, then buffer exchanged into 30 mM Tris-HCl, pH 7.0/2 M urea at 4°C using a Centricon YM-3 (3,000 MW cutoff, Amicon, Beverly, MA, USA). Digestion was initiated with the addition of 1.5 μg trypsin (Sequencing Grade, Boehringer Mannheim, Indianapolis, IN, USA) and incubated overnight at 37°C. The digest was acidified with 30 μL 10% TFA. LC-MS/MS analysis was performed using a Hewlett Packard 1100 HPLC system (Hewlett-Packard, Palo Alto, CA, USA) connected on-line with a 1 : 1 splitter to a Finnigan LCQ iontrap mass spectrometer (Thermoquest, San Jose, CA, USA) as described.[42] Peptides were separated on a C18 1.0×250 mm column (Vydac, Hesperia, CA, USA) using a flow rate of 60 μl/min, with the UV monitored at 215 nm. Solvent A was 0.1% formic acid/H_2O, and solvent B was 0.09% formic acid/H_2O/90% acetonitrile. The gradient consisted of 3% B isocratic for 15 min, a linear gradient to 60% B in 57 min, to 90%B in 15 min, isocratic at 90%B for 15 min, and then to 3% B in 5 min. To determine the amount to load for subsequent runs, an aliquot of the digest ($\sim$8 μg) was analyzed using the 'triple play' feature for data-dependent ion selection and fragmentation (MS, zoom MS, MS/MS) with precursor ion selection over the mass range 400–2000 *m/z*. With the Finnigan Navigator software (Thermoquest, San Jose, CA, USA), an isotope exclusion width of 5 and a dynamic exclusion time of 1.0 min were utilized and a relative collision energy of 40 was set. The remaining digest ($\sim$32 μg) was aliquoted into three samples and analyzed as above but with different ion selection criteria. The ion selection criteria for the triple play utilized one of the mass ranges of 400–615, 585–815, and 785–2000 *m/z* for each analysis. Finnigan.RAW files were converted to .txt files, then fragment ion spectra were searched against non-redundant protein sequence and translations of EST databases.[42,43] Potential peptide matches were visually confirmed.

Reversible cysteine biotinylation of peptide mixture

One-hundred-and-twenty μg sample was vacuum concentrated and resuspended in 1 mL of 40 mM Tris-HCl/pH 7.0/8 M urea/10 mM dithiotreitol, then incubated for 30 min at 37°C. The sample was buffer exchanged into PBS/ 1 mM EDTA/ 2 M urea using a Centricon YM-3 (3000 MW cutoff) at 4°C. Fifty μl of 4 mM EZ-Link ^{TM}Biotin-HPDP (Pierce, Rockford, IL, USA) in DMSO was added and the sample incubated at room temperature for 90 min. To remove excess free Biotin-HPDP the sample was buffer exchanged again into PBS/1 mM EDTA/ 2 M urea, followed by trypsin digestion as above. An 1 mL Immobilized-Avidin column (Pierce, Rockford, IL, USA) was equilibrated with 5 ml PBS/1 mM EDTA. The biotinylated digest was applied to the column and incubated for 30 min at room temperature. The avidin column was washed with 10 mL PBS/1 mM EDTA (yielding the flowthrough fraction). Two mL of PBS/50 mM dithiotreitol was added to elute any bound peptides (yielding the bound fraction). Both the avidin flowthrough fraction and the avidin bound fraction were alkylated (using 10 and 100 mM iodoacetamide respectively) for 30 min at room temperature. The fractions were acidified with formic acid, desalted using an Oasis cartridge (Waters, Milford, MA, USA), vacuum concentrated, and then resuspended in 100 μl 0.1% formic acid. LC-MS/MS analysis was performed using the above listed parameters but with a C18 0.05×150 mm column (MetaChem Technologies Inc., Torrance, CA, USA) and a flow rate of 25 μl/min without splitting directly into the mass spectrometer. Four separate LC-MS/MS runs using separate narrow mass ranges for ion selection (400–575, 565–715, 685–970, and 935–2000 *m/z*) were used for the flowthrough fraction and the bound fraction was analyzed using the single broad mass range (400–2000 *m/z* for ion selection. Spectra were handled as described above.

Immunofluorescence

Mouse embryonic fibroblasts were cultured in the absence or presence of the apoptosis inducer staurosporin (STS; 2 μM, 4 h; Sigma). A rabbit antiserum generated against DDP [37] was used (diluted 1/20) on paraformaldehyde (4% w:v) and picric acid-fixed (0.19% v:v) cells (cultured on 100 μm cover slips; 18 mm $\varnothing$; Superior, Germany), and revealed with a goat anti-rabbit IgG conjugated to phycoerythrine (PE) (Southern Biotechnology, Birmingham, AL, USA). A sheep antiserum specific for SOD2 (Calbiochem, San Diego, CA, USA) was revealed by means of a rabbit anti-sheep IgG fluorescein isothiocyanate (FITC) conjugate (Southern Biotechnology). Cells were counterstained for the detection of hsp60 (mAb H4149 from Sigma, revealed by a goat anti-mouse IgG FITC or PE conjugate; Southern Biotechnology). Confocal microscopy was performed on a Leica TC-SP (Leica Microsystems, Heidelberg, Germany) equipped with an ArKr laser mounted on an inverted Leica DM IFBE microscope with a $63 \times 1{,}32$ NA oil objective.

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