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Proteome-wide Identification of HtrA2/Omi Substrates

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To identify apoptotic targets of HtrA2/Omi, we purified recombinant HtrA2/Omi and its catalytically inactive S306A mutant. Lysates of human Jurkat T lymphocytes incubated with either wild-type recombinant HtrA2/Omi or the S306A mutant were screened using the gel-free COFRADIC approach that isolates peptides covering the N-terminal parts of proteins. Analysis of the 1162 proteins identified by mass spectrometry yielded 15 HtrA2/Omi substrates of potential physiological relevance together holding a total of 50 cleavage sites. Several processing events were validated by incubating purified recombinant HtrA2/Omi with *in vitro* translated substrates or with Jurkat cell lysates. In addition, the generated set of cleavage sites was used to assess the protein substrate specificity of HtrA2/Omi. Our results suggest that HtrA2/Omi has a rather narrow cleavage site preference and that cytoskeletal proteins are prime targets of this protease.

Keywords: COFRADIC • HtrA2 • Omi • apoptosis • substrate

Introduction

The mitochondrial serine protease high-temperature requirement protein A2 (HtrA2/Omi) is expressed from a nuclear gene as a proenzyme of 49 kDa with an N-terminal mitochondrial localization signal that mediates its translocation into the mitochondrial intermembrane space (IMS).[1,2] The mammalian enzyme shares sequence and structural homology with the *Escherichia coli* serine protease HtrA/DegP.[3−5] HtrA/DegP is localized in the periplasmic space, where it controls bacterial thermal and oxidative tolerance by acting as a chaperone at normal temperatures. At elevated temperatures, the chaperone transforms into an active endoprotease that degrades damaged and misfolded proteins.[6−8] Evidence for an evolutionary conservation of this function is provided by the observation that the expression of HtrA2/Omi is upregulated following heat shock of human neuroblastoma SH-SY5Y cells.[9] Moreover, the proteolytic activity of recombinant HtrA2/Omi is significantly elevated upon heat shock.[10] The perinatal death of mice with a targeted deletion of the *HtrA2/Omi* gene substantiates the essential role of this mitochondrial serine protease.[11] The juvenile death of these mice is preceded by a dramatic loss of striatal neurons, resulting in a neurodegenerative disorder with features of Parkinson's disease.[11] Interestingly, the naturally occurring motor neuron deficient (*mnd2*) mice display a similar phenotype, and the underlying cause was found to be a S276C mutation in the gene encoding HtrA2/Omi.[12] As this mutation strongly impairs the proteolytic activity of HtrA2/Omi, the loss of this activity most likely causes the pronounced phenotype of both HtrA2/Omi-deficient and *mnd2* mice. As both *mnd2* and HtrA2/Omi-deficient cells are more susceptible to cytotoxic agents that trigger apoptosis through the intrinsic pathway (e.g., etoposide, tunicamycin, antimycin, and H_2O_2) as compared to wild-type cells, a cytoprotective role involving the proteolytic activity of HtrA2/Omi in the mitochondrial IMS of healthy cells has been suggested.[11,12] In a recent report, two additional mutations in HtrA2/Omi with diminished proteolytic activity (A141S and G399S) have been identified in samples from German patients suffering from Parkinson's disease.[13]

Apoptosis is a genetically programmed form of cell death characterized by stereotypic morphological and biochemical features, including cytoplasmic shrinkage, 'blebbing' of the cell membrane, chromatin condensation, internucleosomal DNA fragmentation, and inhibition of translation.[14] A plethora of apoptotic stimuli converge on the mitochondria and affect their membrane integrity. As a consequence, several death-promoting factors residing in the IMS, including HtrA2/Omi, are released in the cytosol.[15] Although caspases are recognized as the main players in the initiation and execution of apoptosis,[16] several reports have substantiated the role of additional proteases, such as HtrA2/Omi, in the apoptotic process.[17,18] In this respect, antisense- and RNA interference-mediated knockdown of HtrA2/Omi was shown to contribute to a higher resistance against a variety of proapoptotic stimuli in multiple cell

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lines.[1,2,19−21] Moreover, overexpression of full-length HtrA2/Omi sensitized for UV− and staurosporin-induced apoptosis.[1,2,22,23] HtrA2/Omi was shown to sequester several members of the inhibitors of apoptosis (IAP) protein family through its IAP binding motif (IBM), thereby contributing to caspase activation.[1,2,22−25] Subsequently, the IAP proteins were also identified as HtrA2/Omi substrates.[21,26,27] The observation that staurosporin-induced cell death of HeLa cells pretreated with the pan-caspase inhibitors zVAD-fmk or Boc-D-fmk is almost completely blocked by siRNA-mediated knockdown of HtrA2/Omi suggests that HtrA2/Omi contributes to apoptosis through additional mechanisms that are independent of caspase activation.[20] Indeed, overexpression of a HtrA2/Omi mutant that is defective in its IAP-binding activity was still able to induce cell rounding and shrinkage in 293T cells.[2,4,22,23] The observation that caspase-3 deficient MCF7 cells overexpressing the IBM-deficient HtrA2/Omi mutant display the same phenotype underscores the possible existence of a caspase-independent contribution to apoptosis.[1] These morphological changes do not coincide with caspase activity or plasma membrane permeabilization, but require the proteolytic activity of HtrA2/Omi (Vande Walle, unpublished results and ref 22). Moreover, this HtrA2/Omi-induced morphology persists in the presence of the caspase inhibitor XIAP, a dominant negative form of caspase-9 or the pan-caspase inhibitor zVAD-fmk.[1,22] In contrast to the S306A mutant, wild-type HtrA2/Omi elicits cell rounding and cellular shrinkage in Apaf-$1^{-/-}$ and caspase-$9^{-/-}$ MEF cells, further substantiating the importance of the proteolytic activity of HtrA2/Omi for these caspase-independent morphological changes.[1] In line with these results, the proteolytic activity of HtrA2/Omi was recently reported to mediate anoikis of intestinal epithelial cells induced by cell detachment from the extracellular matrix independently of caspase-9 and Smac/Diablo.[28] Finally, the requirement of the proteolytic activity of HtrA2/Omi was confirmed in several studies that used the synthetic HtrA2/Omi inhibitor Ucf-101,[19,29−34] although the latter results could also be attributed to off-target effects of this pharmacological inhibitor.[35]

Despite the wealth of circumstantial evidence on the pleiotropic roles of HtrA2/Omi and its proteolytic activity in apoptosis, little is known about its cytosolic targets, the cleavage of which could account for the observed morphological changes. Indeed, in addition to the IAP proteins, only HS1-Associated Protein X-1 (HAX-1) and the phosphoprotein Ped/Pea-15 have been identified as substrates of HtrA2/Omi during apoptosis.[31,32] To identify new potential substrates of HtrA2/Omi, we generated recombinant wild-type and catalytically inactive S306A HtrA2/Omi to perform a proteome-wide differential combined fractional diagonal chromatography (COFRADIC) analysis.[36] By this means, we identified 50 cleavage sites, allowing us to define the substrate specificity of HtrA2/Omi for native proteins.

Experimental Section

Plasmids. The cDNA encoding mature human HtrA2/Omi was amplified by reverse transcription-PCR from a HepG2 cDNA library using the following primers: HtrA2/Omi-forward, 5′-GGGGTACCATGGCCGTCCCTAGCCCGCCG-3′; HtrA2/Omi-reverse, 5′-TCATTCTGTGACCTCAGGGGTC-3′. The amplified product was digested with *KpnI* and *XbaI*, and the *KpnI*−*XbaI* fragment was cloned into the pEF1-V5-HisA vector (Invitrogen, Carlsbad, CA). The HtrA2/Omi open reading frame was amplified by PCR using the following primers: HtrA2-forward2, 5′-GGGGTACCAATGGCCGTCCCTAGCCCGCCG-3′; HtrA2-reverse2, 5′-TCCCCCCGGGTCATTCTGTGACCTCAGGGG-3′. The amplified product was digested with *KpnI* and *XmaI*, and the *KpnI*−*XmaI* fragment was cloned into the pLT10TH vector[37] in frame with the N-terminal His-tag. The cDNA encoding the catalytically inactive S306A mutant of HtrA2/Omi was generated by overlap PCR technology. The plasmids pOTB7-Vimentin, pOTB7-HADH2, pBluescriptR-KIAA0251, and pCMV-SPORT6-KIAA1967 were obtained from RZPD (Berlin, Germany). Proper construction of all the plasmids was confirmed by DNA sequencing.

Purification of Recombinant HtrA2/Omi. Plasmids encoding wild-type HtrA2/Omi or the S306A mutant were transformed in the *E. coli* strain MC1061 containing a pICA2 plasmid. Exponentially growing *E. coli* bacteria ($A_{600} = 0.5$) were induced for 16 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 20 °C. Cells from 4 L of culture were harvested by centrifugation, resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol, and 300 mM NaCl), and lysed in a French press. Insoluble proteins and cell debris were removed by centrifugation. Bacterial nucleic acids were removed using a DEAE column (Amersham Biosciences, Buckinghamshire, U.K.) equilibrated with buffer A. The flow through was incubated overnight at 4 °C with Ni-NTA beads (20 mL slurry) (Qiagen, Hilden, Germany) in the presence of 5 mM imidazol. The beads were subsequently packed in a column and washed with 3 column vol of buffer B (20 mM Tris-HCl, pH 7.5, 10% glycerol, 300 mM NaCl, and 5 mM imidazol) and 2 column vol of buffer C (20 mM Tris-HCl, pH 7.5, 10% glycerol, 300 mM NaCl, and 20 mM imidazol). His_6-tagged HtrA2/Omi and HtrA2/Omi S306A were eluted from the column with buffer D (20 mM Tris-HCl, pH 7.5, 10% glycerol, 300 mM NaCl, and 100 mM imidazol). The purity of the preparation was checked by SDS-PAGE separation followed by Coomassie blue staining and Western blot analysis using the Penta-His antibody (Qiagen, Hilden, Germany). Selected fractions were pooled and diluted 6 times with 20 mM Tris-HCl, pH 7.5. The samples were then loaded on a Source 15Q anion exchange column (Amersham Biosciences, Buckinghamshire, U.K.), and proteins were eluted using a NaCl gradient ranging from 25 mM to 1 M in a volume of 100 mL (10 column vol) and collected in 7-mL fractions. Wild-type HtrA2/Omi and the S306A mutant behaved identically throughout the purification procedure and eluted from the source 15Q column at a NaCl concentration from ∼350 to ∼600 mM in four 7-mL fractions. Fractions were adjusted to 10% glycerol (v/v) and stored at −80 °C in 1-mL aliquots.

In Vitro β-Casein Cleavage Assay. The proteolytic activity of recombinant purified HtrA2/Omi was monitored by incubating 1−600 nM HtrA2/Omi or HtrA2/Omi S306A with 3 μM β-casein (Sigma-Aldrich, St. Louis, MO) for 30 min at 37 °C in 24 μL of 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 200 mM NaCl, and 5% glycerol. In some experiments, recombinant HtrA2/Omi was pretreated with the pharmacological inhibitor Ucf-101 (EMD Biosciences, San Diego, CA) for 10 min at 37 °C. Reaction products were separated on SDS-PAGE gels and visualized by Coomassie blue staining.

Cell Culture and Induction of Apoptosis. Human Jurkat T cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin (100 Units/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. Before induction of apoptosis, Jurkat cells were resuspended in fresh medium at a density of 500 000 cells/mL. The next day, cells were treated

with 10 μg/mL doxorubicin, 20 μM etoposide, 15 μM camptothecin, or 100 μM cisplatin to induce apoptosis.

COFRADIC Isolation of N-Terminal Peptides. Jurkat cells (10^7) were collected by centrifugation for 5 min at 200*g* at 4 °C, washed twice with cold PBS, and resuspended in 1 mL of ice-cold homogenization buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, supplemented with 1 mM DTT, and a Complete protease inhibitor cocktail tablet (Roche, Mannheim, Germany)). After homogenization by douncing, samples were cleared from membrane fractions by centrifuging at 20 000*g* for 30 min at 4 °C, and the protein concentration was determined using Bio-Rad's protein assay (Bio-Rad, München, Germany). Subsequently, 1 mg of protein extract in a volume of 100 μL was incubated for 1 h at 37 °C with 1 μM recombinant HtrA2/Omi or HtrA2/Omi S306A. Subsequent steps for the sorting and identification of differentially generated N-terminal peptides by COFRADIC were performed as described previously.[36] Briefly, the proteins present in the lysates were reduced and their cysteines alkylated; subsequently, their free amino groups were blocked by acetylation. Following trypsin (sequencing grade modified trypsin, Promega, Madison, WI) digestion, the samples were incubated in either natural or ^{18}O-rich H_2O (95% [$H_2{}^{18}O$] (w/w) pure, ARC Laboratories, AW Apeldoorn, The Netherlands). The differently labeled samples were mixed in a 1:1 ratio and fractionated by reverse-phase (RP) HPLC (primary run). Peptides in each fraction were treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS) to block free α-N-termini of trypsin-generated internal peptides. Note that at this stage, peptides carrying an N-terminal protein part (i.e., N-terminal peptides from either unprocessed proteins or protein fragments generated by proteolytic processing) are not affected by TNBS since their α-amino group was already, either *in vitro* or *in vivo*, blocked. During a replicate secondary RP HPLC run, the TNBS-altered peptides shifted to later elution times, since they acquired an increased hydrophobicity. This procedure was repeated for each of the primary fractions, thus, finally isolating a set of unaltered, N-terminal peptides that do not shift during the two consecutive runs and which were collected for mass spectrometric analysis. Automated LC−MS/MS analysis and peptide identification was done as reported previously.[36]

Immunoblot Analysis. Human Jurkat T cells were washed twice in cold PBS and lysed for 1 min in NP-40 buffer (10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 10% glycerol, supplemented with 0.1 mM PMSF, 200 U/mL aprotinin, 10 mg/mL leupeptin, and 10 mM DTT). For cytosolic HtrA2/Omi and cytochrome *c* detection, cells were permeabilized in cold digitonin 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, supplemented with 0,2 mg/mL digitonin, 0.1 mM PMSF, 200 U/mL aprotinin, 10 mg/mL leupeptin, and 10 mM DTT). This treatment allows selective lysis of the outer cell membrane without affecting the organelle membranes. Samples were cleared from membranes by centrifugation at 20 000*g* for 10 min at 4 °C. Lysates were separated on different percentage of SDS-PAGE gels and transferred to nitrocellulose membrane by semidry blotting in a buffer containing 25 mM Tris-HCl, pH 8.0, 190 mM glycine, and 20% methanol. All further incubations were carried out at room temperature on a platform shaker. Blocking, incubation with antibody, and washing of the membrane were done in PBS supplemented with 0.02% Tween-20 (v/v) and 3% (w/v) nonfat dry milk. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies against mouse, rabbit, and rat immunoglobulin (Amersham Biosciences, Buckinghamshire, U.K.). Immunoreactive proteins were visualized with the enhanced chemiluminescence method (Perkin-Elmer, Boston, MA).

Rabbit polyclonal antiserum against recombinant anti-HtrA2/Omi was prepared at Eurogentec (Herstal, Belgium). The Immunoglobulin G (IgG) fraction was prepared by ammonium sulfate precipitation (50% saturation, 17 000*g* for 10 min at 4 °C), resuspension in binding buffer (20 mM sodium phosphate, pH 7.0), and dialysis at 4 °C against a 1000 volume excess of binding buffer. After clearing by centrifugation, the supernatant was applied onto a HiTrap affinity protein G column (Amersham Biosciences, Buckinghamshire, U.K.) following the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, U.K.). Other primary antibodies used were anti-cytochrome *c* (BD Biosciences, San Jose, CA), anti-caspase-2 (11B4; Millipore, Billerica, MA), anti-tubulin α (B-5-1-2; Sigma, St. Louis, MI), anti-tubulin β (Abcam, Cambridge, U.K.), anti-actin (C-4; MP Biomedicals, Aurora, OH), anti-EF-1α (CBP-KK1; Millipore, Billerica, MA), anti-TIF1β (Abcam, Cambridge, U.K.), and anti-eIF-4G (BD Biosciences, San Jose, CA).

***In Vitro* Transcription/Translation.** pOTB7-Vimentin, pOTB7-HADH2, pBluescriptR-KIAA0251, and pCMV-SPORT6-KIAA1967 plasmids (250 ng each) were used as templates for *in vitro* coupled transcription/translation in a rabbit reticulocyte lysate system according to the manufacturer's instructions (Promega, Madison, WI). For detection of the translation products, ^{35}S-methionine was added to the translation reactions. Translation reactions (2 μL each) were incubated with 1 μM of purified recombinant HtrA2/Omi or HtrA2/Omi S306A in 24 μL of a 50 mM Tris-HCl, pH 7.5, buffer for 1 or 3 h at 37 °C. The resulting cleavage products were analyzed by autoradiography or SDS-PAGE gels.

Results

Production and Characterization of Recombinant HtrA2/Omi. To obtain enzymatically active preparations of HtrA2/Omi, we expressed mature human HtrA2/Omi in *E. coli*. As a negative control, the catalytically inactive S306A mutant was also recombinantly obtained. The recombinant proteins were purified as described in Experimental Section and yielded more than 95% pure material as determined by Coomassie blue staining (Figure 1A). In addition to the major 37-kDa band that represents full-length HtrA2/Omi, anti-His tag Western blot analysis of the wild-type HtrA2/Omi preparations reveals several additional N-terminal fragments, most probably due to autoproteolysis, since they were not detected in the case of the S306A mutant (Figure 1A).

In vitro cleavage assays using β-casein as an artificial substrate for purified recombinant HtrA2/Omi were performed to test the enzymatic activity of the preparations. Wild-type HtrA2/Omi (100 nM) cleaved full-length β-casein to generate the previously reported 15-kDa fragment (Figure 1B).[9,38] To rule out that a co-purified bacterial protease was responsible for the observed cleavage event, wild-type HtrA2/Omi was preincubated with the pharmacological HtrA2/Omi inhibitor Ucf-101.[29] In line with a previous report,[29] 80 μM Ucf-101 completely abolished the cleavage of β-casein (Figure 1C). Moreover, β-casein was not processed when incubated with the enzymatically inactive S306A preparation (Figure 1B).

HtrA2/Omi Is Released in the Cytosol of Apoptotic Jurkat Cells. To investigate HtrA2/Omi expression and localization

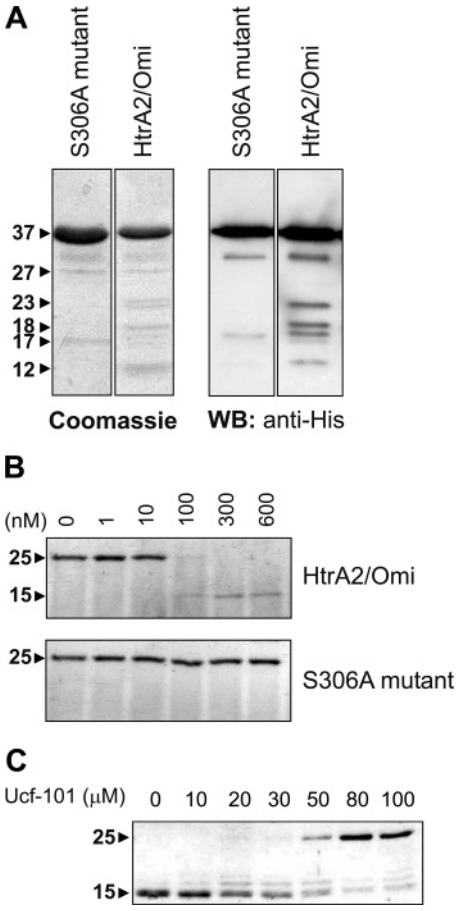

Figure 1. Characterization of purified recombinant HtrA2/Omi. (A) A total of 10 μg of recombinant HtrA2/Omi or HtrA2/Omi S306A mutant (S306A mutant) was separated on SDS-PAGE gel and analyzed by Coomassie blue staining or Western blot analysis using anti-His tag antibody. (B) β-Casein (3 μM) was treated with the indicated concentrations of recombinant purified HtrA2/Omi or HtrA2/Omi S306A (S306A mutant) for 30 min at 37 °C. (C) Recombinant HtrA2/Omi (400 nM) was incubated with 3 μM β-casein in the absence or the presence of the indicated concentrations of Ucf-101.

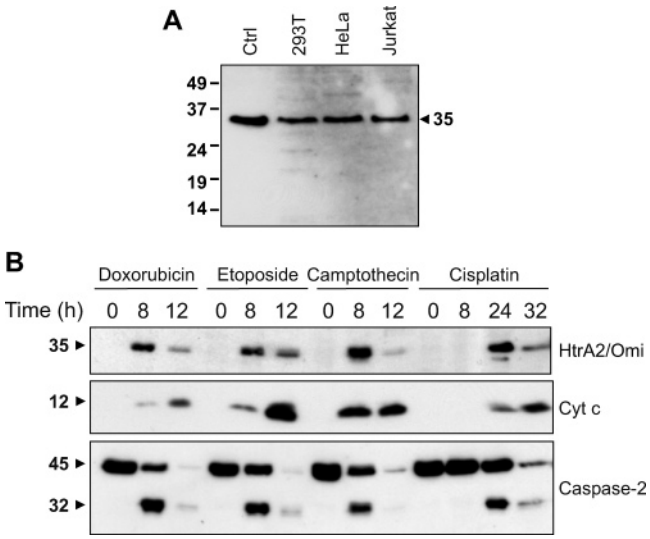

Figure 2. HtrA2/Omi translocates to the cytosol upon an apoptotic stimulus. (A) Western blotting with anti-HtrA2/Omi antibody revealed HtrA2/Omi protein in lysates from 293T, Jurkat, and HeLa cell lines. 293T lysates overexpressing mature HtrA2/Omi in 293T cells was used as a positive control (Ctrl). (B) Human Jurkat T lymphocytes were treated with doxorubicin (10 μg/mL), etoposide (20 μM), camptothecin (15 μM), and cisplatin (100 μM) for the indicated periods of time. The cytosolic extract was analyzed by immunoblotting with anti-HtrA2/Omi (first panel), anti-cytochrome *c* (second panel), and anti-caspase-2 (third panel) antibodies.

during apoptosis, a polyclonal antibody against recombinant HtrA2/Omi was generated. The polyclonal antibody detected by Western blotting in the human cell lines 293T, Jurkat, and HeLa a single protein of ~35 kDa, corresponding to endogenous mature HtrA2/Omi (Figure 2A). The antibodies were specific for HtrA2/Omi, since no cross-reactivity to other proteins was observed by Western blotting and the size of the observed band corresponds to that of overexpressed mature HtrA2/Omi in 293T cells (Figure 2A). It was reportedly demonstrated that HtrA2/Omi is released from the mitochondria into the cytosol upon induction of apoptosis.[1,2,19,22−24] In line with this result, cytosolic release of mitochondrial HtrA2/Omi was observed in Jurkat T cells upon apoptosis induction by a variety of genotoxic stress-inducing agents, such as doxorubicin, etoposide, campthothecin, or cisplatin (Figure 2B). In most cases, the maximal release of HtrA2/Omi in the cytosol preceded the maximal release of cytochrome *c*. For example, cytosolic accumulation of HtrA2/Omi was maximal within 8 h of treatment with doxorubicin, etoposide, or campthothecin (Figure 2B). Parallel Western blot analysis of procaspase-2, a known marker for DNA damage-induced cell death,[39,40] served as a loading control (Figure 2B). These results demonstrate that HtrA2/Omi is released into the cytosol of apoptotic Jurkat T cells and provides a rationale to perform a proteome-wide analysis of potential HtrA2/Omi substrates in lysates of these cells.

Identification of Potential HtrA2/Omi Substrates. To minimize alterations in the conformation of native proteins, we chose to disrupt cells mechanically by shearing rather than lysing them with nonionic detergents. Subsequently, the lysates were coincubated for 1 h at 37 °C with 1 μM recombinant HtrA2/Omi or its catalytically inactive S306A mutant, separately. HtrA2/Omi processing sites were determined using a proteome-wide differential N-terminal peptide analysis by means of the COFRADIC peptide sorting method. Following LC−MS/MS analysis, 1964 peptides were unambiguously identified using the Mascot algorithm,[41] and these finally converged into 1162 different proteins. On the basis of the differential isotope labeling used here, peptides that display as couples of "light" and "heavy" forms spaced by 4 Da are present in both the control setup (inactive HtrA2/Omi) and the protease setup (active HtrA2/Omi) and, thus, do not hint at protein processing. Such peptides were therefore not considered for further data interpretation. On the other hand, peptides exclusively found in the proteome digest of HtrA2/Omi-treated cell lysates (thus represented by a single isotopic peptide envelope) were further regarded as hinting at HtrA2/Omi substrates. In this way, 15 potential HtrA2/Omi substrates were identified, and together, these proteins harbor 50 HtrA2/Omi cleavage sites (Table 1). Interestingly, six potential substrates are main cytoskeletal proteins, including actin, tubulin α, tubulin β, and vimentin. Furthermore, we identified two protein translation regulators: eukaryotic translation initiation factor 4 gamma 1 (eIF-4G1) and the elongation factor 1-alpha (EF-1α). Two proteins are

Table 1. Internally Located α-N-Acetylated Peptides Generated by Recombinant HtrA2/Omi[a]

accession	protein name	identified peptide	site	M_r (kDa)	M_r fragments (kDa)
P68133	Actin*	Ac-K_{18}AGFAGDDAPR$_{28}$	SGLV-K	42	2−40
		Ac-T_{106}EAPLNPKANR$_{116}$	PVLL-T		12−30
		Ac-E_{107}APLNPKANR$_{116}$	VLLT-E		12−30
		Ac-A_{220}LDFEQEMATAASSSSLEKSYELPDGQVITIGNER$_{254}$	LCYV-A		24−17
Q71U36	Tubulin α*	Ac-G_{57}AGKHVPR$_{64}$	FSET-G	50	6−44
		Ac-V_{66}FVDLEPTVIDEVR$_{79}$	VPRA-V		7−43
		Ac-F_{67}VDLEPTVIDEVR$_{79}$	PRAV-F		7−43
		Ac-D_{69}LEPTVIDEVR79	AVFV-D		7−43
		Ac-L_{70}EPTVIDEVR$_{79}$	VFVD-L		7−43
		Ac-I_{93}TGKEDAANNYAR$_{105}$	PEQL-I		10−40
		Ac-F_{138}HSFGGGTGSGFTSLLMER$_{156}$	GFLV-F		15−35
		Ac-A_{201}FMVDNEAIYDICR$_{214}$	HSDC-A		22−28
		Ac-D_{205}NEAIYDICR$_{214}$	AFMV-D		22−28
		Ac-E_{254}FQTNLVPYPR$_{264}$	VDLT-E		28−22
		Ac-T_{292}NACFEPANQMVKCDPR$_{308}$	VAEI-T		32−18
		Ac-N_{329}AAIATIKTKR$_{339}$	PKDV-N		37−14
		Ac-G_{354}INYQPPTVVPGGDLAKVQR$_{373}$	GFKV-G		39−11
		Ac-Y_{408}VGEGMEEGEFSEAR$_{422}$	FVHW-Y		45−5
		Ac-E_{414}EGEFSEAR$_{422}$	GEGM-E		46−4
P07437	Tubulin β-2*	Ac-S_{25}DEHGIDPTGTYHGDSDLQLDR$_{46}$	WEVI-S	50	3−47
		Ac-S_{48}VYYNEATGGKYVPR$_{62}$	LDRI-S		5−44
		Ac-D_{67}LEPGTMDSVR$_{77}$	AVLV-D		7−42
		Ac-A_{102}KGHYTEGAELVDSVLDVVR$_{121}$	GNNW-A		11−39
		Ac-E_{108}GAELVDSVLDVVR$_{121}$	GHYT-E		12−38
		Ac-T_{136}HSLGGGTGSGMGTLISKIR$_{156}$	GFQL-T		15−35
		Ac-G_{140}GGTGSGMGTLLISKIR$_{156}$	THSL-G		15−35
		Ac-T_{218}TPTYGDLNHLVSATMSGVTTCLR$_{241}$	TLKL-T		24−26
		Ac-Y_{222}GDLNHLVSATMSGVTTCLR$_{241}$	TTPT-Y		24−26
		Ac-S_{230}ATMSGVTTCLR$_{241}$	NHLV-S		25−25
		Ac-R_{241}FPGQLNADLR$_{251}$	TTCL-R		26−24
		Ac-A_{254}VNMVPFPR$_{262}$	LRKL-A		28−22
		Ac-H_{264}FFMPGFAPLTSR$_{276}$	FPRL-H		29−21
		Ac-V_{293}FDAKNMMAACDPR$_{306}$	LTQQ-V		32−18
Q99867	Tubulin β-4q*	Ac-H_{137}SLGGGTGSGMGTLLLSKIR$_{156}$	FQLT-H	48	15−33
		Ac-G_{140}GGTGSGMGTLLLSKIR$_{156}$	THSL-G		15−33
Q9BUF5	Tubulin β-6*	Ac-A_{364}STFIGNSTAIQELFKR$_{380}$	GLKM-A	50	40−10
P08670	Vimentin*	Ac-G_{40}SALRPSTSR$_{49}$	TYSL-G	54	4−49
O94788	RALDH 2	Ac-A_{260}FTGSTEVGKLIQEAAGR$_{277}$	IDKI-A	57	28−29
Q99714	HADH2*	Ac-V_{59}FAPADVTSEKDVQTALALAKGKFGR$_{84}$	GNNC-V	27	6−21
Q04637	eIF-4G1*	Ac-S_{95}YPASQGAYYIPGQGR$_{110}$	PSQI-S	176	10−166
P68104	EF-1α*	Ac-Y_{254}KIGGIGTVPVGR$_{266}$	LQDV-Y	50	28−22
Q13263	TIF1-β*	Ac−K_{184}YTKDHTVR$_{192}$	HQRV-K	88	18−71
P27708	CAD	Ac−A_{1101}YTDGDLER$_{1109}$	AMNV-A	243	120−123
O75351	VPS4B	Ac−K_{155}FPHLFTGKR$_{164}$	ILPI-K	49	17−32
Q8N163	KIAA1967	Ac−A_{133}ALGQKQGILGAQPQLIFQPHR$_{154}$	LLHV-A	103	14−89
		Ac−S_{170}HTLHLSHLNR$_{180}$	LFQT-S		18−85
Q86XE2	KIAA0251	Ac−A_{240}NAGTAAVGHTDKIGR$_{255}$	LLLV-A	55	27−28
O43464	HtrA2/Omi	Ac−S_{212}GDTYEAVVTAVDPVADIATLR$_{233}$	VRLL-S	37	11−27
		Ac−Q_{267}NTITSGIVSSAQR$_{280}$	PFAL-Q		16−21
		Ac−I_{270}TSGIVSSAQR$_{280}$	LQNT-I		17−20

[a] Peptides identified in the proteome-wide COFRADIC analysis. The complete list of identified internally α-N-acetylated peptides is shown. Protein names are identified by using the SIB BLAST network service, and the accession numbers are according to the Swiss-Prot/TrEMBL database. Information on the sequence of the identified peptide, its location within the protein, and the amino acids preceding the identified peptide is given. Ac denotes an α-N-acetyl group, and an asterisk indicates processing events validated by immunoblotting or *in vitro* transcription/translation experiments. The predicted molecular mass (M_r) of the full-length substrates and proteolytic fragments generated by one HtrA2/Omi cleavage event are indicated in integers.

known dehydrogenases, namely, retinal dehydrogenase 2 (RALDH2) and hydroxyacyl-coenzyme A dehydrogenase type II (HADH2). Other known proteins that were identified are the transcription intermediary factor 1-beta (TIF-1β), the carbamoyl-phosphate synthetase aspartate transcarbamoylase-dihydroorotase (CAD) that mediates *de novo* pyrimidine synthesis, and the vacuolar sorting protein 4b (VPS4B), which is associated with intracellular protein trafficking. Finally, two potential HtrA2/Omi substrates (KIAA1967 and KIAA0251) were identified, the function of which has not yet been well-characterized, although KIAA0251 contains a decarboxylase domain (http://www.sanger.ac.uk/Software/Pfam/) suggesting it may be involved in carboxylic acid metabolism. The nucleoprotein KIAA1967 was initially cloned from a homozygously deleted region on chromosome 8p21 in breast and other cancers, although no function has been attributed yet for the protein product it encodes.[42] Interestingly, a caspase-generated N-terminal fragment of KIAA1967, which is also known as Deleted in Breast Cancer-1 or DBC-1, was recently reported to translocate from the nucleus to the cytosol and mitochondria of apoptotic HeLa cells, where it induces mitochondrial clustering and matrix condensation.[43] In addition to these 15 substrates of potential physiological relevance, the differential degradomics analysis identified 3 cleavage fragments originating from wild-type HtrA2/Omi (Table 1). As discussed earlier, these fragments most probably correspond to the autoproteolytic fragments present in the recombinant preparation, as the size of the latter correlates with the expected size based on the

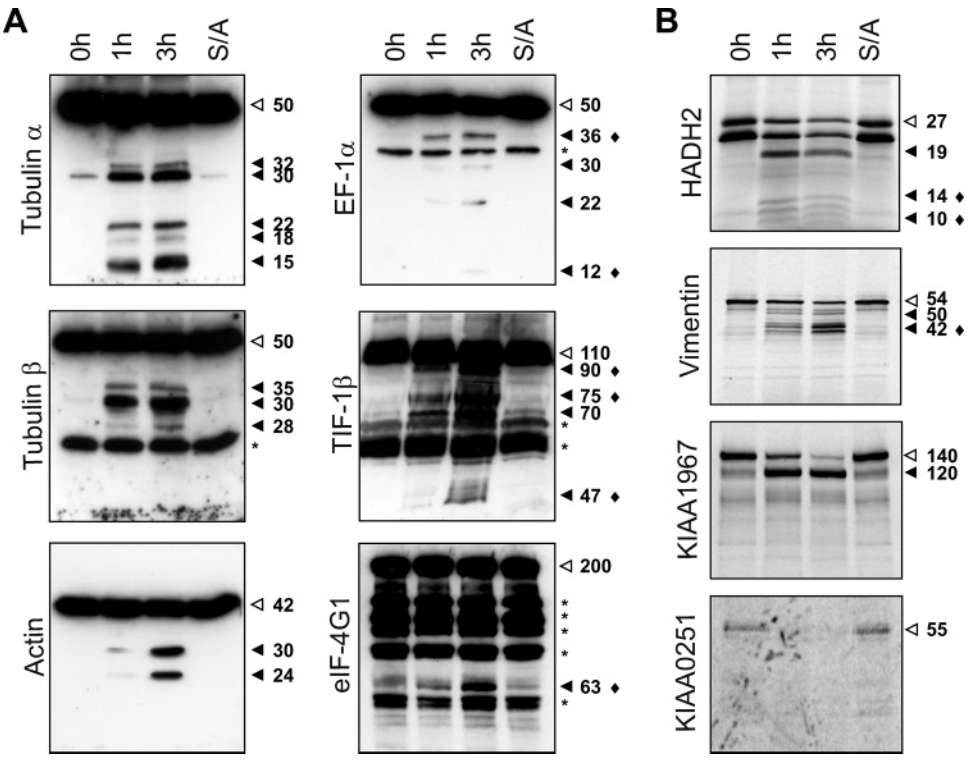

Figure 3. Validation of identified potential HtrA2/Omi substrates. (A) Western blot analysis of Jurkat cell lysates left untreated or incubated separately with 1 μM recombinant HtrA2/Omi and HtrA2/Omi S306A (S/A) for the indicated time points revealed the cleavage of tubulin α, tubulin β, actin, EF-1α, TIF-1β, and eIF-4G1. An open arrow denotes the full-length protein, and an asterisk indicates a-specific bands. (B) HADH2, vimentin, KIAA1967, and KIAA0251 were *in vitro* transcribed and translated in the presence of ^{35}S-methionine and left untreated or treated with 1 μM recombinant HtrA2/Omi or HtrA2/Omi S306A mutant (S/A) for the indicated times. Autoradiography shows HtrA2/Omi dependent cleavage of the radiolabeled proteins.

cleavage site identified in the COFRADIC analysis (Figure 1A and Table 1). Notably, several substrates listed in Table 1 harbor multiple cleavage sites, the P1 position of which differs by only one to six amino acids. To analyze whether these cleavage events could be attributed to specific cleavage by HtrA2/Omi or instead were due to an aspecific aminopeptidase activity, we used the WebLogo presentation of the sequence alignment of peptides harboring the identified cleavage sites (http://weblogo.berkeley.edu/) and compared the P1 cleavage site specificity originating from these sites with that obtained from substrates with unique HtrA2/Omi cleavage sites. This comparative analysis (data not shown) revealed no significant differences in cleavage site specificity, suggesting that most likely HtrA2/Omi is directly responsible for all observed processing events.

Validation of Identified Targets. To confirm the cleavage events identified in the differential COFRADIC analysis, we performed immunoblot analysis of cell lysates. These experiments indicated actin, tubulin-α and -β, EF-1α, TIF-1β, and eIF-4G1 cleavage fragments in Jurkat cell lysates incubated with wild-type HtrA2/Omi (Figure 3A). Furthermore, the size of the proteolytic fragments was in accordance with the theoretical size based on the cleavage sites identified in the COFRADIC analysis. An overview of these calculated masses is presented in Table 1. Interestingly, additional cleavage fragments were observed for EF-1α, TIF-1β, and eIF-4G1 (marked with a diamond symbol in Figure 3A). For example, two extra fragments of approximately 36 and 12 kDa were present on a Western blot with a monoclonal antibody against EF-1α (Figure 3A). Since we failed to detect any cleavage fragments using certain antibodies (HADH2, vimentin) and because of a lack of suitable antibodies in other cases (KIAA1967 and KIAA0251), we coincubated ^{35}S-radiolabeled proteins with recombinant HtrA2/Omi to study whether these proteins can be cleaved by HtrA2/Omi (Figure 3B). Again, the size of the cleavage fragments of HADH2 and vimentin was in accordance with the expected HtrA2/Omi cleavage sites as identified in the COFRADIC experiment (Figure 3B). Additional cleavage fragments that could be detected on the autoradiographs are marked with a diamond symbol in Figure 3B. KIAA1967, which migrates at a higher-than-expected size on autoradiograms, is cleaved by HtrA2/Omi to generate a fragment with an apparent size of 120 kDa (Figure 3B). Processing of KIAA0251 was confirmed by the disappearance of the full-length proteins upon co-incubation with wild-type HtrA2/Omi, although no distinct cleavage products of the latter substrate were readily visible (Figure 3B). Taken together, these results confirm the cleavage of 10 out of 15 identified HtrA2/Omi substrates and the size of most cleavage fragments corresponds to the processing sites identified in the differential COFRADIC analysis (Table 1).

Determination of the Protein Cleavage Site Specificity of HtrA2/Omi. The optimal peptide cleavage site motif for HtrA2/Omi has been characterized previously using a combinatorial peptide library approach.[10] To determine whether the specificity of the identified target proteins corresponds with the reported peptide substrate specificity, we analyzed the primary sequence selectivity in the 50 protein cleavage sites identified here (Figure 4A). Noteworthy, cysteine was almost completely absent in all of the determined cleavage sites (Table 1 and Figure 4A). Clustering of the amino acids based on the

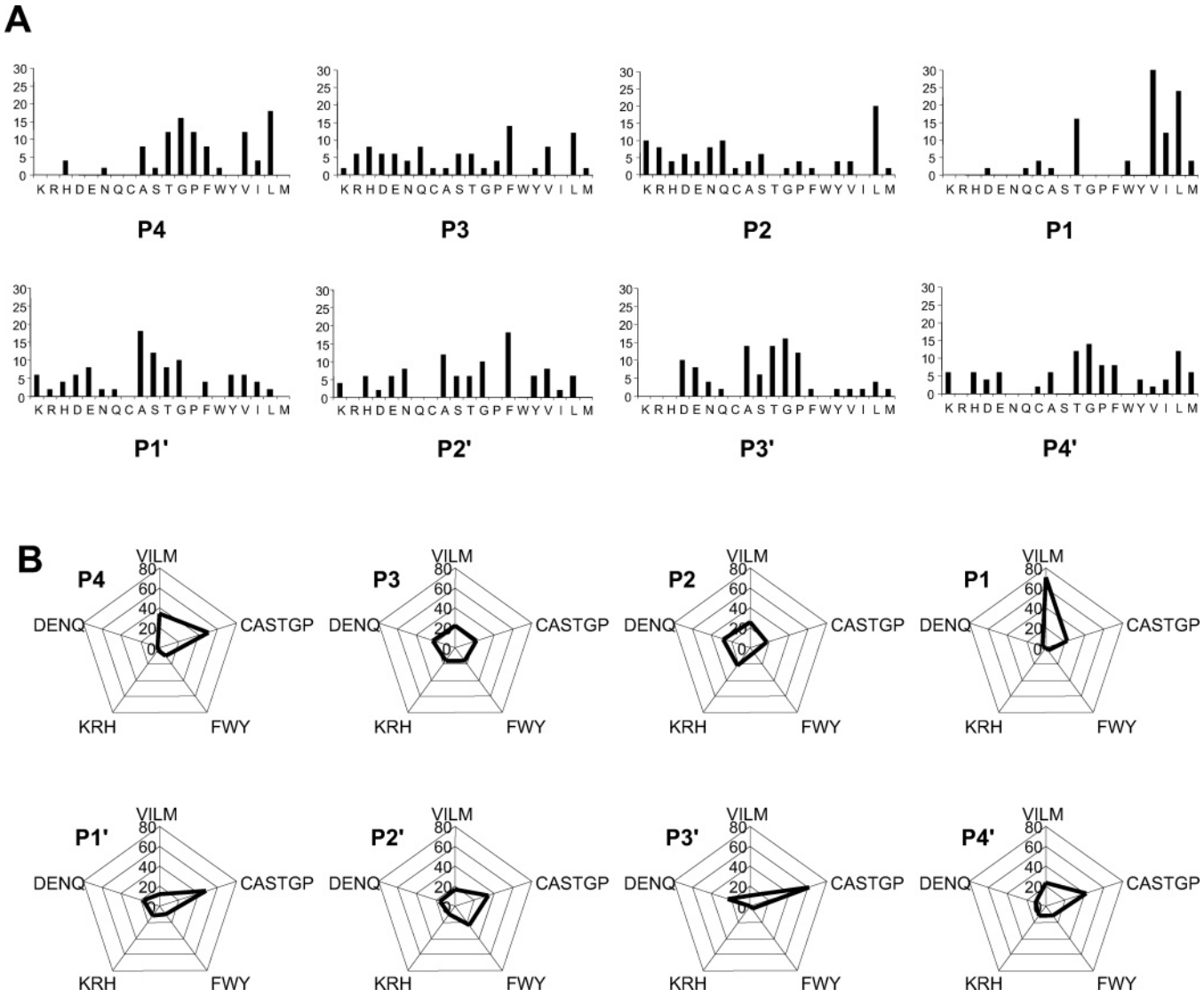

Figure 4. Protein substrate cleavage site specificity of HtrA2/Omi. (A) Values represent the amino acid percentage found at positions C-terminal (P1′, P2′, P3′, P4′) and N-terminal (P1, P2, P3, P4) to the HtrA2/Omi cleavage site. (B) Radar graph of the cleavage site specificity of HtrA2/Omi. The amino acids have been grouped based on their characteristics of side chain residues (aliphatic, small, aromatic, basic, and acidic).

characteristics of the side chain residues (respectively, VILM, CASTGP, FWY, KRH, and DENQ) demonstrates that HtrA2/Omi displays a striking preference for aliphatic residues (in 70% of the cleavage sites) at the P1 position, with a secondary selection for threonine (Figure 4). At the P2, P1′, and P2′ position, the substrate specificity profile shows some selection for leucine, alanine, and phenylalanine, respectively, in nearly 20% of the cleavage sites (Figure 4A). While no pronounced specificity was apparent at the P3 positions, we found certain selectivity (62%) for small amino acids in the P3′ position (Figure 4B). Finally, our results show a secondary selection for small or hydrophobic amino acids in the P4 and P4′ positions (Figure 4B). Altogether, these results indicate that HtrA2/Omi prefers to cleave after aliphatic residues, while small or hydrophobic amino acids preferentially occupy the P4 and the four positions C-terminal to the cleavage site.

Discussion

The serine protease HtrA2/Omi was originally identified as an Mxi-2 interaction partner in a yeast two-hybrid screen.[38] Soon, it became clear that HtrA2/Omi is a mitochondrial factor that is released into the cytosol during apoptosis elicited by a wide variety of stimuli (TNF, anti-Fas, TRAIL, DNA damaging agents, staurosporin, UV).[1,2,19,22−24] Cytosolic HtrA2/Omi sequesters members of the IAP protein family through its N-terminal IAP binding motif.[1,2,22−24] Moreover, IAP proteins are also targets of HtrA2/Omi-mediated proteolysis.[21,26,27] These mechanisms contribute eventually to enhanced caspase activity. In this respect, HtrA2/Omi promotes apoptosis in a caspase-dependent fashion. As HtrA2/Omi is a serine protease, it may also contribute to the rounding of cells[1,2,4,22,23] or other apoptotic processes. In this respect, the proteolytic activity of HtrA2/Omi was recently shown to be essential for anoikis induced by cell detachment from the extracellular matrix.[28]

Besides the IAP proteins, only HAX-1 and the phosphoprotein Ped/Pea-15 have been identified as HtrA2/Omi substrates during apoptosis.[31,32] To better characterize the protease function of HtrA2/Omi, we performed a proteome-wide study to identify potential HtrA2/Omi substrates. Through mass spectrometric identification of 1964 peptides, belonging to more

than 1000 different proteins, 50 peptides were attributed specifically to HtrA2/Omi protease activity. These 50 cleavage events collapse onto 15 potential HtrA2/Omi substrates (Table 1). Subsequently, we validated 10 of these 15 proteins as HtrA2/Omi substrates of potential physiological relevance by immunoblotting or autoradiography (Figure 3). In agreement with the cell rounding phenotype that is observed upon overexpression of catalytically active HtrA2/Omi and the importance of the proteolytic activity of HtrA2/Omi in anoikis, we identified and validated the cytoskeleton proteins actin, tubulin α, tubulin β, and vimentin as HtrA2/Omi targets. Furthermore, two proteins involved in protein translation, that is, eukaryotic translation initiation factor 4 gamma 1 (eIF-4G1) and elongation factor 1-alpha (EF-1α), were also cleaved by HtrA2/Omi (Table 1). The identification of components of the translation machinery as possible HtrA2/Omi targets is in line with the abrogation of *de novo* protein synthesis during apoptosis, coinciding with caspase-dependent cleavage of eIF-4G1.[44,45] The dehydrogenase L-3-hydroxyacyl-coenzyme A dehydrogenase HADH2 or ERAB (ER-associated amyloid β (Aβ) binding protein) is another interesting HtrA2/Omi substrate. This protein was identified as an Aβ interaction partner and constitutes a direct molecular link between Aβ and mitochondrial toxicity.[46,47] In addition to HADH2/ERAB, β-amyloid precursor protein was recently demonstrated to be an *in vivo* mitochondrial substrate of HtrA2/Omi during homeostasis.[48] The link between HtrA2/Omi and Alzheimer's disease is further substantiated by the observation that presenilin may regulate HtrA2/Omi protease activity.[49] Finally, the less-characterized proteins KIAA1967 and KIAA0251 have recently been associated with apoptosis.[43] A caspase-generated cleavage fragment of KIAA1967 has been shown to induce mitochondrial clustering and matrix condensation in apoptotic HeLa cells,[43] whereas KIAA0251 interacts with the endoplasmic reticulum (ER)-resident membrane protein Bap29, a factor known to be essential for caspase-8 activation at the ER.[50] Altogether, the identified and validated substrates of HtrA2/Omi suggest an involvement of this protease in the apoptotic process at the level of the cytoskeleton, the translation initiation complex, and organelle dismantling. Interestingly, most of the identified HtrA2/Omi substrates are also known targets of caspases, including actin, tubulin α, vimentin, eIF-4G1, TIF-1β, and KIAA1967, indicating their substantial fate during cell death.[36,51] Additionally, KIAA0251 was recently identified as a caspase substrate in daunorubicin-treated AML cells (Van Damme et al. unpublished results).

Martins and colleagues reported that HtrA2/Omi favors aliphatic residues in position P1, while the choice for basic residues at the P2 and P3 positions, alanine or serine in P1′, or aromatic residues at P2′ is of secondary importance.[10] These authors used a combinatorial peptide library approach to define the optimal peptide cleavage site motif for HtrA2/Omi.[10] The 50 protein cleavage sites that we have identified here allowed us to examine whether the reported cleavage site specificity for peptide substrates matches the selection of target proteins. Our analysis revealed that aliphatic residues are present at the P1 position in 70% of the cleavage sites (Figure 4B). Our approach revealed a secondary specificity of HtrA2/Omi for threonine at the P1 position, which was absent from the analysis of the reported combinatorial peptide library covering the P4 to P1 sites.[10] At the P1′ position, alanine is selected most strongly, with a secondary selection for serine (Figure 4A). Furthermore, the P2′ position harbors the aromatic phenylalanine in nearly 20% of the cleavage sites (Figure 4A). Noteworthy, the reported selectivity of HtrA2/Omi in peptide substrates for basic residues at the P2 and P3 positions was absent in our set of protein cleavage sites (Figure 4B). On the other hand, we found a certain selectivity (62%) for small amino acids in the P3′ position, while this position seemed to be completely aspecific in the study of Martins and colleagues (Figure 4B and ref 10).

This apparent discrepancy in cleavage site specificity between peptide and protein substrates of HtrA2/Omi may reflect the involvement of additional structural elements in the protein substrates that interact with the protease and influence its topological targeting and enzymatic activity. Indeed, the selection of target proteins was suggested to be partially dependent on interactions with the C-terminal PDZ domain of HtrA2/Omi.[10] This hypothesis is underscored by the X-ray structure of the HtrA2/Omi trimer, which shows that the PDZ domains restrict access to the protease domains.[4] Interaction of the PDZ domain with a binding partner may trigger a conformational change in the flexible L3 loop and a reorientation of the so-called 'activation domain' formed by loops L1, L2, and LD, thus, exposing the enzymatic cleft of the protease.[4] This model is supported by the observation that deletion of the PDZ domain of HtrA2/Omi results in increased protease activity against β-casein and several peptide substrates.[4,10] In line with these reports, we observed that substrate processing was significantly enhanced in the absence of the C-terminal PDZ domain (Supporting Information Figure 1). Variations in the natural frequency of occurrence of amino acids in endogenous proteins may be an additional parameter that may help explain the differences between peptide and protein cleavage site specificities. Indeed, glycine, alanine, serine, and threonine are known to be overrepresented in vertebrate protein sequences, while other amino acids such as cysteine and tryptophan appear less frequently. This variation in occurrence is normally absent from a systematic analysis of peptide sequences in a combinatorial library approach. Taking the frequency of occurrence into account, the HtrA2/Omi specificity for aromatic residues at the P2′ site increases from 24% up to 37%, while the preference for small amino acids at the P3′ position decreases from 62% down to 48%. Noteworthy, the previously reported HtrA2/Omi cleavage sites in the protein substrates cIAP1[21] and β-amyloid precursor protein[48] fit remarkably well with our analysis, thus, providing additional support for the HtrA2/Omi cleavage site specificity determined here. Moreover, our results are in accordance with the reported preferential cleavage of peptide substrates between valine or isoleucine in P1, alanine or serine in the P1′, and aromatic residues in the P2′ position[10] Finally, a similar specificity for aliphatic amino acids at the P1 position was found for the bacterial HtrA2/Omi homologue HtrA/DegP, suggesting that this selectivity has been evolutionarily conserved.[52]

Altogether, we have identified and validated several HtrA2/Omi substrates of potential physiological relevance in a systematic proteome-wide degradomics approach. HtrA2/Omi-mediated cleavage of these proteins may affect cytoskeletal reorganizations, inhibit protein translation and modulate organelle stress. Furthermore, the identification of 50 cleavage sites in these protein substrates enabled us to analyze the protein substrate specificity of this evolutionarily conserved serine protease.

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Supporting Information Available: Figure showing the kinetics of vimentin cleavage by wild-type and PDZ-deficient HtrA2/Omi. This material is available free of charge via the Internet at http://pubs.acs.org.

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