Identification of the novel substrates for caspase-6 in apoptosis using proteomic approaches

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Apoptosis, programmed cell death, is a process involved in the development and maintenance of cell homeostasis in multicellular organisms. It is typically accompanied by the activation of a class of cysteine proteases called caspases. Apoptotic caspases are classified into the initiator caspases and the executioner caspases, according to the stage of their action in apoptotic processes. Although caspase-3, a typical executioner caspase, has been studied for its mechanism and substrates, little is known of caspase-6, one of the executioner caspases. To understand the biological functions of caspase-6, we performed proteomics analyses, to seek for novel caspase-6 substrates, using recombinant caspase-6 and HepG2 extract. Consequently, 34 different candidate proteins were identified, through 2-dimensional electrophoresis/MALDI-TOF analyses. Of these identified proteins, 8 proteins were validated with *in vitro* and *in vivo* cleavage assay. Herein, we report that HAUSP, Kinesin5B, GEP100, SDCCAG3 and PARD3 are novel substrates for caspase-6 during apoptosis. [BMB Reports 2013; 46(12): 588-593]

INTRODUCTION

Apoptosis is a programmed cell death in multicellular organisms, employed for the development and maintenance of homeostasis, and removal of damaged cells (1). It has been demonstrated that caspases play a pivotal role in apoptosis (2). Caspases cleave their substrates after aspartic acid at P1 sites, by using cysteine residue in the catalytic site (3). P4-P1 residue in the target proteins determines the substrate specificity of caspases (4, 5). In apoptosis, caspases are classified into initiator caspases and executioner caspases (6). Once the extrinsic death signal is given by the ligand-induced activation of death receptors, initiator caspase-8 and caspase-10 are activated by dimerization (7, 8). The death signal is delivered by an internal factor, such as DNA damage, and the release of cytochrome c from the mitochondria induces the formation of the apoptosome. Initiator caspase-9 is recruited to the apoptosome complex, and activated by dimerization (9). In turn, this activated initiator caspases activate the executioner caspases. The activated executioner caspases cleave their specific target proteins (10). Executioner caspases include caspase-3, -7 and caspase-6. Among them, it has been well known that caspase-3 cleaves a large set of substrates, and plays a major role in apoptosis (11, 12). Caspase-7, sharing the high sequence homology with caspase-3, recognizes the same cleavage site of caspase-3 (4, 13), suggesting that caspase-3 and -7 have redundant roles (11). However, caspase-6 has somewhat unique roles as an executioner in apoptosis. Caspase-6 prefers leucine or valine at the P4 position, whereas caspase-3 and -7 prefer aspartate at the P4 position in target substrates (14, 15). Therefore, caspase-6 has unique substrates that are not cleaved by either caspase-3 or caspase-7, such as lamin A/C (16), special AT-rich binding protein-1 (SATB1) (17), the neurodegenerative disease proteins Huntingtin (18), amyloid precursor protein (19), Presenilin 1, 2 (20), and DJ-1 (21). The most distinctive feature of caspase-6 is that it is activated by caspase-3 and -7 (22), and inversely activates caspase-3 and -7 (23). Also, caspase-6 acts as an initiator caspase, by processing initiator caspase-8 and -10 during apoptosis (22, 24). Although the role of caspase-6 has been proposed in a number of reports, its substrates and its function are still unclear during apoptosis.

Thus, to better understand the biological roles of caspase-6 during apoptosis, we carried out proteomic analyses to seek for novel sets of caspase-6 substrates. In our present study, we report novel putative 34 substrates for caspase-6, and show that 5 of them, HAUSP, Kinesin5B, GEP100, SDCCAG3, and PARD3, are directly cleaved by caspase-6 during apoptosis.

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RESULTS AND DISCUSSION

Screening of putative substrates for caspase-6 by proteomic approaches

To seek for novel caspase-6 substrates, we performed two-dimensional gel electrophoresis based proteomics analyses (Fig. 1A). HepG2, a human hepatoma cell line was used, since HepG2 cells have not been studied to screen for caspase substrates (14). To increase the possibility of finding more candidates in 2DE analyses, HepG2 cell extract was fractionated into cytosol, membrane and nucleus fractions, and each subcellular fraction was incubated with either recombinant active caspase-6 or catalytic mutant substituted 163-cysteine into serine in active site (inactive caspase-6). Then, incubated proteins were separated by isoelectric focusing and SDS-PAGE (Fig. 1A). The experiments were repeated three times, independently, to establish reproducibility. By comparing two-dimensional gel images of active caspase-6 and inactive caspase-6 mutant, appeared or disappeared protein spots were selected. Consequently, we found different 39 spots in cytosolic (Fig. 1B), 33 spots in membrane (Fig. 1C), and 62 spots in nucleus fraction (Fig. 1D). These protein spots were identified by MALDI-TOF- MS analysis. To obtain reliable results, proteins that have >95% confidence level in the Mascot program (Matrix Science) were selected. To determine whether the identified candidates were novel or not, we carried out a database search at The CASBAH (http://bioinf.gen.tcd.ie/casbah) and the CutDB, a proteolytic event database (http://cutdb.burnham.org), and a literature search at Pubmed. Finally, we obtained a list of novel candidates of caspase-6 substrates (Table 1). It is worth noting that the representative caspase-6 substrates, such as lamin A/C, vimentin, actin, tubulin, and ezrin, were identified in our screening system indicating that our screening method was functioning properly (Table 1).

HAUSP, Kinesin5B, GEP100, SDCCAG3 and PARD3 are directly cleaved by caspase-6 *in vitro*

In order to confirm whether the candidate proteins listed in Table 1 would be cleaved by caspase-6, we performed the *in vitro* cleavage assay. HepG2 cell extracts were incubated with either active caspase-6, or catalytic mutant caspase-6. The cleavage was detected by western blotting, using the specific antibodies. Eight candidate proteins (HAUSP, Kinesin 5B, GEP100, SDCCAG3, PARD3, CLK3, ADH4, and MDH1), of which commercial antibodies are available, were tested. Lamin A/C, a well known caspase-6 substrate, was used as a positive control. As a result, the level of HAUSP, Kinesin 5B, GEP100, SDCCAG3 and PARD3 reduced only when active caspase-6 was treated. This indicates that these proteins were cleaved directly by caspase-6, according to Lamin A/C cleavage (Fig. 2A). However, these cleavages were blocked in the presence of caspase-6 inhibitor, Z-VEID-FMK (Fig. 2A). Therefore, these results indicate that HAUSP, Kinesin5B, GEP100, SDCCAG3 and PARD3 are directly cleaved by caspase-6. On the other hand, CLK3, ADH4 and MDH1 were not cleaved by caspase-6, even
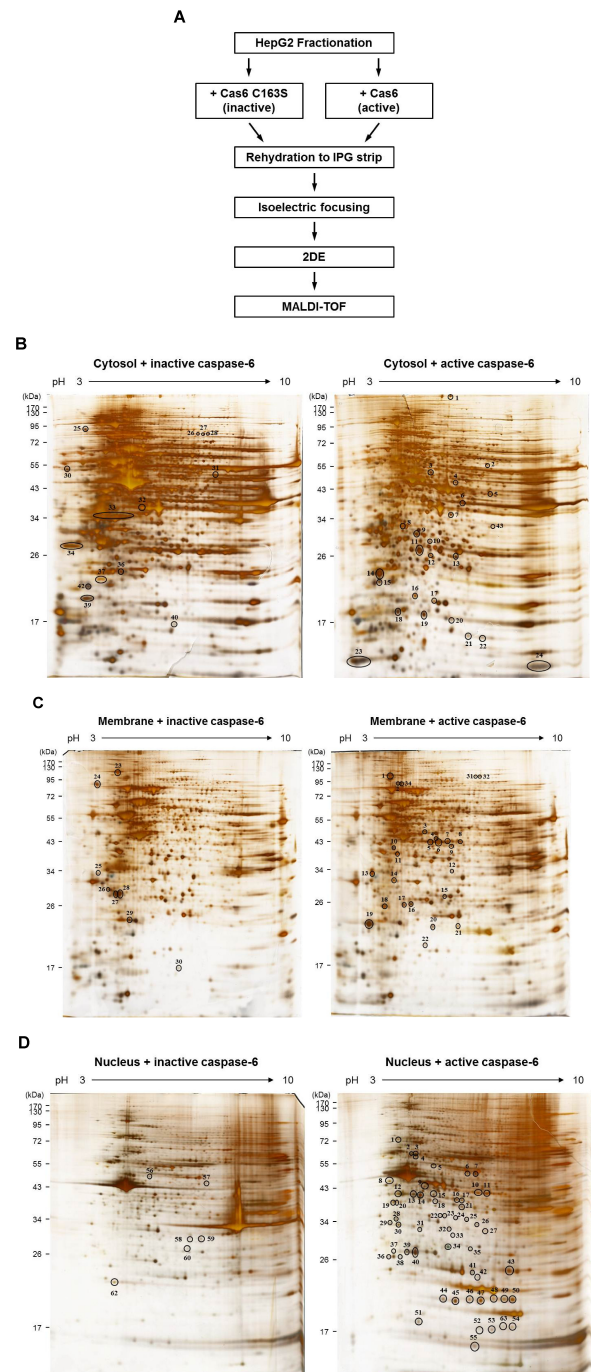

Fig. 1. (A) The workflow for screening to identify novel caspase-6 substrates by proteomics approaches. (B-D) 2-DE gel images showing the differentially displayed spots. (B) Cytosol fractions, (C) membrane fractions, or (D) nucleus fractions, treated with either active caspase-6 or inactive casspase-6, were analyzed using 2-DE. The experiments were repeated for three times, independently. The representative gel images are shown.

Table 1. Identified proteins from 2DE-Proteomics and MALDI-TOF-MS analysis

Fraction	Spot no.	Identified protein description	Symbol	Accession no.	Mascot score[a]	Matched peptide	Sequence coverage (%)	Molecular weight (Da)	pI
Cytosol	2	Dual specificity protein kinase CLK3	CLK3	gi‚Äâ194097436	67	13	28	74,267	9.94
	5	Tryptophanyl-Trna Synthetase	WARS	gi‚Äâ50513261	66	13	27	43,586	7.12
	9	Chain A, Human Ubiquitous Kinesin Motor Domain	Kinesin5B	gi‚Äâ157830287	66	9	40	36,792	5.88
	11	Actin, cytoplasmic 1	Beta-actin	gi‚Äâ4501885	748	24	29	42,052	5.29
	12	Alpha-actin	Alpha-actin	gi‚Äâ178027	118	2	7	42,480	5.23
	21	GTP-binding protein Rit1 isoform 2	Rit1	gi‚Äâ5902050	67	10	55	25,357	9.2
	24	Serologically defined colon cancer antigen 3	SDCCAG3	gi‚Äâ55961008	66	9	69	18,921	5.74
Membrane	2	IQ motif and Sec7 domain 1	GEP100	gi‚Äâ119584552	66	15	24	121,073	9.2
	8	Herpesvirus associated ubiquitin-specific protease	HAUSP	gi‚Äâ1545952	70	20	20	129,274	5.33
	10	Tubulin, gamma complex associated protein 2 variant	TUBGCP2	gi‚Äâ62898904	67	16	27	103,011	6.38
Nucleus	3	Alpha-tubulin	Alpha-tubulin	gi‚Äâ340021	90	14	35	50,804	4.94
	5	Antigen identified by monoclonal antibody Ki-67	Ki-67	gi‚Äâ119569563	76	32	14	360,616	9.48
	6	Translin-associated factor X interacting protein 1	TSNAX	gi‚Äâ119603582	67	14	25	72,374	5.11
	9	Dynein, axonemal, heavy polypeptide 1	DNAH1	gi‚Äâ119585621	70	32	10	497,514	5.62
	10	Gephyrin	Gephyrin	gi‚Äâ16605466	67	14	33	80,410	5.25
	11	Olfactomedin-like protein 1 precursor	OLFL1	gi‚Äâ284172520	66	11	41	46,378	8.29
	12	Alcohol dehydrogenase 4	ADH4	gi‚Äâ825623	67	9	41	41,094	8.25
	13	Actin, beta, partial	Beta-actin	gi‚Äâ14250401	70	13	52	41,321	5.56
	15	Peroxisomal bifunctional enzyme isoform 1	EHHADH	gi‚Äâ68989263	66	12	25	80,072	9.24
	16	PR domain containing 5	PRDM5	gi‚Äâ119625672	66	12	36	50,017	9.49
	20	Regulatory factor X, 5 (influences HLA class II expression)	RFX5	gi‚Äâ122889236	67	13	31	63,473	9.41
	21	KIAA0524SARM protein	-	gi‚Äâ7711002	67	12	26	76,260	8.58
	22	Partitioning-defective 3-like protein splice variant c	PARD3	gi‚Äâ18874468	66	16	19	125,435	8.57
	24	Dynein, axonemal, heavy polypeptide 1	DNAH1	gi‚Äâ119585622	67	21	12	280,703	5.74
	25	Kinesin-like protein KIF16B	KIF16B	gi‚Äâ41327691	74	23	19	152,488	5.86
	25	Ezrin	Ezrin	gi‚Äâ46249758	64	17	25	69,313	5.94
	26	Cytoplasmic linker associated protein 1	CLASP1	gi‚Äâ119615659	66	15	23	102,275	9.53
	27	Adenosine deaminase	ADA	gi‚Äâ1197210	67	11	42	35,335	5.6
	28	Herpesvirus associated ubiquitin-specific protease	HAUSP	gi‚Äâ1545952	112	26	24	129,274	5.33
	29	Alpha tubulin	Alpha-tubulin	gi‚Äâ109093209	72	13	40	46,767	5.01
	30	Bullous pemphigoid antigen 1	BPAG1	gi‚Äâ27923959	69	34	12	374,544	6.38
	31	Vimentin	VIM	gi‚Äâ62414289	70	16	27	53,676	5.06
	33	Inter-alpha-trypsin inhibitor family heavy chain-related protein	IHRP	gi‚Äâ1483187	97	21	28	103,549	6.51
	34	Lamin A/C	LMNA	gi‚Äâ21619981	193	27	40	53,222	6.03
	35	Lamin A/C	LMNA	gi‚Äâ21619981	107	19	30	53,222	6.03
	37	GT mitochondrial solute carrier protein homologue; putative, partial	-	gi‚Äâ386960	66	9	37	38,645	9.9
	38	MIF4G domain-containing protein isoform 2	MIF4GD	gi‚Äâ23510352	66	7	33	29,941	5.29
	39	Actin, cytoplasmic 2-like	ACTG1	gi‚Äâ354468985	111	17	62	28,478	5.2
	40	Actin, beta	Beta-actin	gi‚Äâ14250401	126	19	44	41,321	5.56
	43	Glutaryl-Coenzyme A dehydrogenase	GCDH	gi‚Äâ119604731	67	11	38	47,923	8.73
	47	Malate dehydrogenase 1B	MDH1	gi‚Äâ89886456	71	13	22	59,013	5.85
	49	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	ATP5A	gi‚Äâ15030240	66	13	28	59,886	9.07
	52	Protein FAM73A	FAM73A	gi‚Äâ38348384	67	13	33	71,930	5.42
	56	Rab GDP dissociation inhibitor beta isoform 1	GDI2	gi‚Äâ6598323	67	10	44	51,087	6.11
	57	WD repeat domain 18	WDR18	gi‚Äâ12804481	67	11	28	47,960	6.22
	58	Coiled-coil domain-containing protein 148	CCDC148	gi‚Äâ76779830	66	8	42	33,401	7.63
	63	ATP/GTP binding protein-like 3	AGBL3	gi‚Äâ22658305	67	14	31	73,571	7.22

[a]proteins scores are significant at 95% confidence level.

though twice the amounts of caspase-6 were used (Fig. 2B), implying that CLK3, ADH4 and MDH1 are not direct substrates for caspase-6.

Candidate proteins were proteolytically processed by caspases during apoptosis

Then we performed the *in vivo* cleavage assay to verify whether the candidates would be cleaved *in vivo* during apoptosis. HeLa

cells were treated with staurosporine (STS), which induces apoptosis by inhibiting cellular protein kinase (25). Apoptosis was confirmed by observing of PARP cleavage (Fig. 3A and 3B), which is a well-established apoptosis hallmark, and is also a caspase-3 and caspase-7 substrate (26, 27). To examine the specificity of caspases, three different caspase inhibitors (Z-VEID-FMK; caspase-6 inhibitor, Z-DEVD-FMK; caspase-3/-7 inhibitor and Z-VAD-FMK; pan-caspase inhibitor) were treated together with STS. In STS stimulated HeLa cells, all candidate proteins (HAUSP, Kinesin5B, GEP100, SDCCAG3, and PARD3) were processed (Fig. 3A). In the presence of Z-VEID-FMK and STS, the cleavage of Kinesin5B and GEP100 was blocked, and recovered to the level of DMSO treated (Fig. 3A). Meanwhile, the cleavage of HAUSP, SDCCAG3, and PARD3 was inhibited slightly. Upon Z-DEVD-FMK and STS treatment, the cleavages of Kinesin5B, GEP100, and SDCCAG3 were inhibited completely, but HAUSP and PARD3 were still cleaved, but less than STS alone (Fig. 3A). Upon STS and Z-VAD-FMK treatment, the cleavage of all candidate proteins was inhibited completely (Fig. 3A). Accordingly, these data suggest that HAUSP, Kinesin5B, GEP100, SDCCAG3, and PARD3 are cleaved somewhat, not only by caspase-6, but also by other caspases, and their cleavages seem not to be mutually exclusive in vivo.

We also checked the rest of the candidates that were not cleaved directly by caspase-6, in the same way (Fig. 3B). CLK3 and ADH4 were proteolytically processed upon STS treatment, but these cleavages were slightly blocked by Z-VEID-FMK or Z-DEVD-FMK, and completely blocked by Z-VAD-FMK. Meanwhile the cleavage of MDH1 was almost completely inhibited by either Z-VEID-FMK or Z-DEVD-FMK. Taken together, CLK3, ADH4, and MDH1 are processed during apoptosis, but they are not direct substrates for caspase-6.

Among the identified caspase-6 substrates, HAUSP is a deubiquitinating enzyme that removes ubiquitin from p53 and MDM2 (28). HAUSP plays a role in cell survival, proliferation and apoptosis, through regulating the p53 level (28). It has been reported that HAUSP is processed in a caspase-dependent manner during thymocyte apoptosis (29). In our study, we demonstrate that HAUSP is directly processed by caspase-6 in human cells, the cervical cancer cell HeLa (Fig. 2A and 3A) and hepatocarcinoma cell HepG2 (data not shown), indicating that HAUSP is caspase-6 specific substrate, and is not cell type specific. Another identified caspase-6 substrate, Kinesin 5B is microtubule associated motor protein transporting vesicles along the microtubule (30). It has been reported that kinectin, kinesin receptor protein, is cleaved by caspase, and the transport system becomes destroyed during apoptosis (31). Based on our results, caspase-6 indeed cleaves kinesin 5B transporting cellular cargoes, as well as cytoskeletal protein, such as tubulin and actin, suggesting that caspase-6 might be involved in the disruption of intracellular vesicle transport process, by inactivating kinesin 5B.

In conclusion, we identified 34 putative caspase-6 substrates, through 2-dimesional electrophoresis/MALDI-TOF analyses.
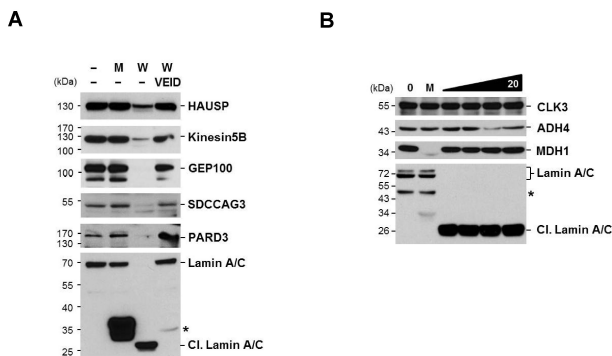

Fig. 2. Cleavage of caspase-6 substrate candidates was confirmed by in vitro cleavage assay. (A) HepG2 extract was incubated with active caspase-6 (10 U) or inactive capsase-6 (10 U) in the presence or absence of 30 μM Z-VEID-FMK. The reaction mixture was analyzed by immunoblotting with the specific antibodies against (A) HAUSP, Kinesin5B, GEP100, SDCCAG3, PARD3 and Lamin A/C. (B) HepG2 extract was incubated with increasing amount of active caspase-6 (5, 10, 15, 20 U) or inactive capsase-6 (20 U). The reaction mixture was analyzed by immunoblotting with the specific antibodies against CLK3, ADH4, MDH1, and Lamin A/C (Cl, cleaved Lamin A/C). Lamin A/C cleavage was used as a positive control. The asterisk indicates a nonspecific band. The representative blot of at least two independent experiments is shown. 1 U is the amount of enzyme required to cleave 1nmole of caspase substrate completely in 1 hr.
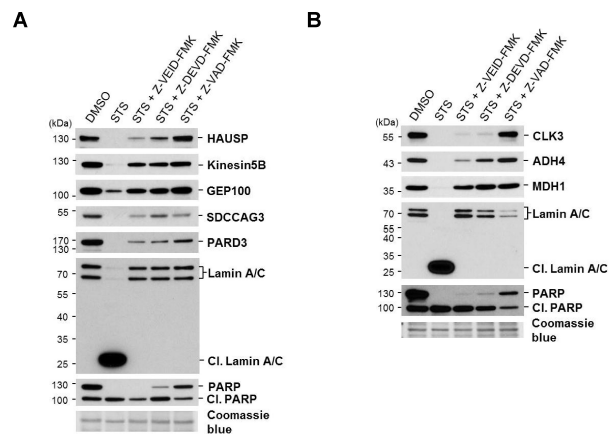

Fig. 3. Cleavage of caspase-6 substrate candidates was confirmed by in vivo cleavage assay. HeLa cells were treated with 1 μM STS for 24 hr in the presence or absence of the 100 μM caspase inhibitors (Z-VEID-FMK; caspase-6 inhibitor, Z-DEVD-FMK; caspase-3/-7 inhibitor, Z-VAD-FMK; pan-caspase inhibitor) pretreated to cells for 3 hr. After 24 hr, cells were harvested and lysed. Cell lysates were analyzed by immunoblotting with the specific antibodies against (A) HAUSP, Kinesin5B, GEP100, SDCCAG3, PARD3, Lamin A/C (Cl, cleaved Lamin A/C), and PARP (Cl, cleaved PARP); and (B) CLK3, ADH4, MDH1, Lamin A/C, and PARP. A coomassie blue stain of PVDF membrane was the loading control. The representative blot of at least two independent experiments is shown.

Furthermore, we confirmed that HAUSP, Kinesin 5B, GEP100, SDCCAG3 and PARD3 are directly cleaved by caspase-6 during apoptosis. These newly identified substrates seem to be involved in membrane trafficking, cell polarization, and p53 stability, suggesting that our results may provide a clue to understanding the roles of caspase-6 in apoptosis.

MATERIALS AND METHODS

Cell culture
HeLa and HepG2 were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA) in a humidified atmosphere of 5% CO_2 in air, at 37°C. HepG2 cells were fractionated using Qproteome Cell Compartment Kit (QIAGEN, USA), according to the manufacture's instruction.

Antibodies and reagents
Anti-PARP (#9542), anti-Lamin A/C (#2032), and anti-HAUSP (#3277) were purchased from Cell Signaling (USA). Anti-ADH4 (ab83819), anti-CLK3 (ab54683), anti-Kinesin5B (ab42492), anti-PARD3 (ab40769), and anti-SDCCAG3 (ab97666) were from Abcam (USA). Anti-GEP100 (G4798) was from Sigma. Anti-MDHC (sc-166879) was from Santa Cruz (USA). Staurosporine (#1048-1) was from BioVision (CA). Z-VEID-FMK (FMK006), Z-DEVD-FMK (FMK004), and Z-VAD-FMK (FMK001) were purchased from R&D System (USA).

Cloning and mutagenesis of caspase-6
Δ23 caspase-6, prodomain deleted mutant, was subcloned to pET21a(+), using *BamH* I and *Xho* I sites. This construct was mutated using the *QuikChange Site-Directed* Mutagenesis Kit (Stratagene, CA), according to the manufacture's instructions, with the following oligonucleotides: Caspase-6 Forward C163S 5′-TTTATCATTCAGGCAAGTCGGGGAAACCAGCAC-3′, Caspase-6 Reverse C163S 5′-GTGCTGGTTTCCCCGACTTGC CTGAATGATAAA-3′. The constructs were confirmed by sequencing at the Solgent (Daejeon, Korea).

Expression and purification of recombinant caspase-6
Recombinant Δ23 caspase-6 and Δ23 caspase-6 C163S with C-terminal His-tag were expressed and purified from *E. coli* BL21 codon plus (DE3) RIL (stratagene, CA), using affinity chromatography. In brief, cells were lysed in the lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0) with 0.5% sarcosine and EDTA free protease inhibitor (Roche, Germany). The lysate was clarified by centrifuging at 13,000 rpm, 4°C for 15min and the supernatant was incubated with Ni-NTA agarose beads (QIAGEN, USA). After extensive washing, bound proteins were eluted with the lysis buffer containing 250 mM imidazole. Protein concentration was determined by the Bradford (Bio-Rad, USA) protein assay.

2-DE analysis and MALDI-TOF-MS
The protein samples were dissolved in 9 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.003% bromophenol blue, and 2% IPG buffer pH3-10 (GE healthcare, USA). The dissolved samples were applied onto immobilized 18 cm, pH 3-10 gradient strips (GE healthcare, USA). Strips were rehydrated, and isoelectric focusing was performed in a stepwise fashion (0 : 01 h at 300 V; 1 : 30 h at 3,500 V; 13 : 00 hr at 3,500 V). After the focused strips were equilibrated with DTT and iodoacetamide equilibration solution for 15 min respectively, the second-dimensional gel electrophoresis was performed on 10% SDS-PAGE. The gels were silver-stained, using PlusOne Silver Staining Kit, Protein (GE healthcare, USA), according to the manufacturer's instructions. For mass spectrometry analysis, silver stained proteins were excised from the gels. The spots were destained and digested with trypsin, and the peptides from the spot were analyzed by MALDI-TOF, at Yonsei Proteome Research Center (Seoul, Korea). The database search was performed at The CASBAH and CutDB. The CASBAH (The CAspase Substrate DataBAse Homepage) is an online database containing all of the reported mammalian caspase substrates (32). CutDB is an online proteolytic event database that provides the list of protease and corresponding substrates and cleavage sites (33).

In vitro cleavage assay
HepG2 cells were lysed using NP40 lysis buffer (137 mM NaCl, 20 mM Tris-HCl pH 8.0, 10% Glycerol, 1% NP40, 2 mM EDTA) containing protease inhibitor (Roche, Germany). Cell lysates were incubated with either recombinant Δ23 caspase-6 protein or Δ23 caspase-6 C163S protein, at 37°C for 24 hr. Reactions were stopped by adding Laemmli buffer, and were boiled at 100°C for 5 min.

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