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 caspas (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 apoptosisosome. Initiator caspase-9 is recruited to the apoptosisosome 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 focussing 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 cytosol (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
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Table 1. Identified proteins from 2DE-Proteomics and MALDI-TOF-MS analysis

| Fraction | Spot no. | Identified protein description | Symbol | Accession no. | Mascot score | Matched peptide | Sequence coverage (%) | Molecular weight (Da) | pL |
|----------|----------|--------------------------------|--------|---------------|--------------|-------------------|----------------------|---------------------|----|
| Cytosol  | 2        | Dual specificity protein kinase CLK3 | CLK3   | gi|194097436 | 67 | 13 | 28 | 74,267 | 9.94 |
|          | 5        | Tryptophanyl-tRNA Synthetase | WARS   | gi|30513261 | 66 | 13 | 27 | 43,586 | 7.12 |
|          | 9        | Chain A, Human Ubiquitous Kinesin | Kinesin5B | gi|157830287 | 66 | 9 | 40 | 36,792 | 5.88 |
|          | 11       | Actin, cytoplasmic 1 | Beta-actin | gi|4501885 | 748 | 24 | 29 | 42,052 | 5.29 |
|          | 12       | Alpha-actin | Alpha-actin | gi|178027 | 118 | 2 | 7 | 42,480 | 5.23 |
|          | 21       | mitochondrial protein RIL isoform 2 | RIL | gi|3902050 | 67 | 10 | 55 | 25,357 | 9.2 |
| Membrane | 8        | IQ2 motif and Sec7 domain 1 | CEP100 | gi|119584552 | 66 | 15 | 24 | 121,073 | 9.2 |
|          | 10       | Tubulin, gamma complex associated protein 2 variant | TUBGCP2 | gi|62899904 | 67 | 16 | 27 | 103,011 | 6.38 |
| Nucleus  | 3        | Alpha-tubulin | Alpha-tubulin | gi|1340021 | 90 | 14 | 35 | 50,804 | 4.94 |
|          | 4        | Antigen identified by monoclonal antibody Ki-67 | Ki-67 | gi|119569563 | 76 | 32 | 14 | 360,616 | 9.48 |
|          | 6        | Translin-associated factor X interacting protein 1 | TSNAX | gi|119603582 | 67 | 14 | 25 | 72,374 | 5.11 |
|          | 9        | Dynemin, axonemal, heavy polypeptide 1 | DNAH1 | gi|119585620 | 70 | 32 | 10 | 497,514 | 5.62 |
|          | 10       | Gephyrin | Gephyrin | gi|16605466 | 67 | 14 | 33 | 80,410 | 5.25 |
|          | 11       | Olactomembrin-like protein 1 precursor | OLF1 | gi|26417220 | 66 | 11 | 41 | 46,378 | 8.29 |
|          | 12       | Alcohol dehydrogenase 4 | ADH4 | gi|825623 | 67 | 9 | 41 | 41,094 | 8.25 |
|          | 13       | Actin, beta, partial | Beta-actin | gi|14230401 | 70 | 13 | 52 | 41,321 | 5.56 |
|          | 15       | Peroxisomal bifunctional enzyme isoform 1 | PBEF1 | gi|68989263 | 66 | 12 | 25 | 80,072 | 9.24 |
|          | 16       | PR domain containing 5 | PRD5 | gi|11962562 | 66 | 12 | 36 | 50,017 | 9.49 |
|          | 20       | Regulatory factor X, 5 (influences HLA class II expression) | RFX5 | gi|122889236 | 67 | 13 | 31 | 63,473 | 9.41 |
|          | 21       | KIAA0742SARM1 protein | - | gi|7711002 | 67 | 12 | 26 | 76,260 | 8.58 |
|          | 22       | Partitoning-defective 3-like protein splice variant c | PARD3 | gi|18874468 | 66 | 16 | 19 | 125,435 | 8.57 |
|          | 24       | Dynemin, axonemal, heavy polypeptide 1 | DNAH1 | gi|119585622 | 67 | 21 | 12 | 280,703 | 5.74 |
|          | 25       | Kinesin-like protein KIF16B | KIF16B | gi|43132791 | 74 | 23 | 19 | 152,488 | 5.86 |
|          | 26       | Extlin | Extlin | gi|46249758 | 64 | 17 | 25 | 69,313 | 5.94 |
|          | 27       | Cytoplasmic linker associated protein 1 | CLASP1 | gi|119615659 | 66 | 15 | 23 | 102,275 | 9.53 |
|          | 28       | Adenosine deaminase | ADA | gi|1197210 | 67 | 11 | 42 | 35,353 | 5.6 |
|          | 29       | Herpesvirus associated ubiquitin-specific protease | - | gi|1545952 | 112 | 26 | 24 | 129,274 | 5.33 |
|          | 30       | Alpha tubulin | Alpha-tubulin | gi|109093209 | 72 | 13 | 40 | 46,767 | 5.01 |
|          | 31       | Bilirubin peroxidase 1 | BFAP1 | gi|27923939 | 69 | 34 | 12 | 374,544 | 6.38 |
|          | 32       | Vimentin | VIM | gi|62414289 | 70 | 16 | 27 | 53,676 | 5.06 |
|          | 33       | Inter-alpha-trypsin inhibitor family heavy chain-related protein | IHRP | gi|1483187 | 97 | 21 | 28 | 103,549 | 6.51 |
|          | 34       | Lamina A | LMNA | gi|21619981 | 193 | 27 | 40 | 53,222 | 6.03 |
|          | 35       | Lamina A | LMNA | gi|21619981 | 107 | 19 | 30 | 53,222 | 6.03 |
|          | 37       | GTP mitochondrial solute carrier protein homologous; putative, partial | - | gi|386060 | 66 | 9 | 37 | 38,645 | 9.9 |
|          | 38       | MIF-4C domain-containing protein isoform 2 | MIF4C2 | gi|23510352 | 66 | 7 | 33 | 29,941 | 5.29 |
|          | 39       | Actin, cytoplasmic 2-like | ACTG1 | gi|354468985 | 111 | 17 | 62 | 28,478 | 5.2 |
|          | 40       | Actin, beta | Beta-actin | gi|14230401 | 126 | 19 | 44 | 41,321 | 5.56 |
|          | 41       | Glutathione-Cysteine A dehydrogenase | GCDH | gi|11964731 | 67 | 11 | 38 | 47,923 | 8.73 |
|          | 47       | Malate dehydrogenase 1B | MDH1 | gi|89886456 | 71 | 13 | 22 | 59,013 | 5.85 |
|          | 49       | ATP synthase, H+ transporting | ATP5A | gi|15030240 | 66 | 13 | 28 | 59,886 | 9.07 |
|          | 52       | Protein FAM73A | FAM73A | gi|38348384 | 67 | 13 | 33 | 71,930 | 5.42 |
|          | 56       | Rab GDP dissociation inhibitor beta isoform 1 | GDI2 | gi|6928323 | 67 | 10 | 44 | 51,087 | 6.11 |
|          | 57       | WD repeat domain 18 | WDR18 | gi|1280481 | 67 | 11 | 28 | 47,960 | 6.22 |
|          | 58       | Colins-alpha isoform containing protein 14B | CDS14B | gi|76778930 | 66 | 8 | 42 | 33,401 | 7.63 |
|          | 63       | ATP7GTP binding protein-like 3 | AGBL3 | gi|22658305 | 67 | 14 | 31 | 73,571 | 7.22 |

*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
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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 caspase-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, and PARD3. Meanwhile, the cleavage of HAUSP, SDCCAG3, and PARD3 was inhibited slightly. Upon Z-DEVD-FMK and STS treatment, the cleavage of Kinesin5B, GEP100, and SDCCAG3 were inhibited completely, but HAUSP and PARD3 were still cleaved, but less than STS alone (Fig. 3A). On STS and Z-VEID-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.

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) 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 cleavage of Kinesin5B, GEP100, and SDCCAG3 were inhibited completely, but HAUSP and PARD3 were still cleaved, but less than STS alone (Fig. 3A). STS and Z-VEID-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. 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 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 kinase, 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-dimensional electrophoresis/MALDI-TOF analyses.
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% CO₂ in air, at 37°C. HepG2 cells were fractionated using Qproteome Cell Compartment Kit (QIAGEN, USA), according to the manufacturer’s instruction. The constructs were confirmed by sequencing at the Solgent (Daejeon, Korea).

Caspase-6 Reverse C163S 5’-GTGCTGGTTTCCCCGACTTGC 3’. The constructs were confirmed by sequencing at the Solgent (Daejeon, Korea).

Antibodies and reagents
Anti-Parp (#9542), anti-Lamin A/C (#20323), 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 NaH₂PO₄, 500 mM NaCl, pH 8.0) with 0.5% sarcosine and equilibrated with DTT and iodoacetamide equilibration solution for 10 min. 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). 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 trypsins, 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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