Genome-wide screening and identification of novel proteolytic cleavage targets of caspase-8 and -10 *in vitro*

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Abstract. Apoptosis executed by the mammalian caspase family plays a fundamental role in cellular homeostasis. Deregulation of this process is associated with several human diseases. The multimerization of ligand-induced death receptors results in the recruitment of the death inducing signaling complex and autocatalytic activation of initiator caspases, including caspase-8 and -10. However, it is still unclear how initiator caspases trigger and control the early apoptotic signaling pathways, partly because the downstream proteolytic cleavage targets of the initiator caspases are not completely known. Although it is known that a number of proteins are cleaved by various members of the caspase family, the identification of specific cleavage substrates of the initiator caspases 8 and 10, has been hindered by a lack of systematic and broadly applicable strategies for substrate identification. In the present study we constructed a mouse cDNA library and used it to perform a systematic, genome-wide screen for novel *in vitro* substrates of caspase-8 and -10. From this, we successfully identified six putative caspase substrates, including five novel proteins (ABCF1, AKAP1, CPE, DOPEY1 and GOPC1) that may be targeted specifically by the initiator caspases 8 and 10 during the early stages of apoptosis. These findings may provide useful information for elucidating the apoptotic signaling pathways downstream of the death receptors.

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Introduction

Apoptosis is essential in the regulation of cellular homeostasis, and its deregulation is associated with various human diseases including cancers, autoimmune diseases, neurodegenerative diseases and immune deficiencies (1,2). The caspases are an evolutionarily conserved family of cysteine proteases involved in diverse cellular functions, including apoptosis (3-5). To date, 14 human and murine caspases have been identified and characterized.

Based on structural characteristics, the caspases can be separated into two major categories: those with a long prodomain (caspase-1, -2, -4, -5, -8, -9, -10, -11, and -12) and those with a short prodomain (caspase-3, -6, -7, and -14). The long prodomains of caspases mediate homotypic protein interactions, allowing these caspases to interact with other proteins. In contrast, caspases with a short prodomain are activated upon proteolytic cleavage by other caspases (1-5). The caspases can also be separated into two distinct functional categories based on their cellular activities (1-5). Caspase-1, -4, -5, and -11 are involved in cytokine maturation and inflammatory responses. The remaining family members are primarily involved in apoptotic signaling pathways: the initiator caspases (caspase-2, -8, -9, -10, and -12) function early in apoptotic signaling pathways and are capable of activating the downstream effector caspases (caspase-3, -6, -7, and -14), either directly or indirectly. Upon

activation by an initiator caspase, the effector caspases, executioners of the apoptotic program, cleave specific cellular substrates to bring about cell death.

It is well established that the multimerization of ligand-induced death receptors results in the formation of the death inducing signaling complex (DISC) and recruitment of initiator caspase-8 and -10 (3,5,6-8). The recruitment and oligomerization of caspase-8 and -10 to the DISC result in their autocatalytic activations and are critical for initiating cell death signaling pathways. Our understanding of early apoptotic signaling pathways, will require the identification of the downstream proteolytic cleavage targets of the initiator caspases. Although a number of structural and signaling proteins are cleaved by members of the caspase family during apoptosis (3,5,9), the identification of specific substrates of initiator caspase-8 and -10 has been hindered by a lack of systematic and broadly applicable strategies. Given the absence of such methods and the growing number of caspase family members, it is likely that the majority of apoptotic caspase targets have yet to be identified. In this report, we describe a method to identify caspase substrates directly and rapidly using labeled protein pools derived from cDNA library pools (7). With this genome-scale approach we identified six putative substrates of caspase-8 and -10, including five novel caspase substrates.

Materials and methods

Construction of a mouse cDNA library and preparation of ^{35}S-labeled protein pools. The cDNA library was constructed from 5 μg of total RNA isolated from mouse liver using the Smart cDNA Synthesis Kit (Clontech, USA) according to the manufacturer's instructions. cDNAs were then inserted into *Eco*RI and *Xho*I sites of pCS2. Pools of cDNAs were transcribed and translated *in vitro* using the TnT Coupled Reticulocyte Lysate System (Promega, USA) in the presence of ^{35}S-methionine (Perkin Elmer, USA).

In vitro proteolytic cleavage assays. ^{35}S-labeled proteins obtained from *in vitro* transcription/translation reaction were incubated for 1 h at 37˚C with a reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM DTT, and 0.1 mM EDTA) or 10 units of recombinant and active human caspase-8 or -10 (Calbiochem, USA). Cleavage reactions were stopped by the addition of 4X SDS sample buffer and boiled for 5 min. The protein products were separated by SDS-PAGE and visualized by autoradiography.

Screening of individual positive cDNA clones and identification of putative caspase substrates. When a protein pool was confirmed positive in the *in vitro* caspase cleavage assay, the corresponding cDNA pool was progressively subdivided and re-examined in the same manner until a single positive cDNA clone was isolated. Positive clones were sequenced (Bionics, Korea) and compared to known sequences by searching the NCBI, Ensembl, Harvester (11), and Scansite (12) databases. Harvester, a meta-search engine for gene and protein information, searches 16 major databases and prediction servers. Scansite identifies short protein sequence motifs that are recognized by modular signaling domains, are phosphorylated by protein kinases, or that mediate specific interactions with protein or phospholipid ligands.

Results and discussion

Systematic screening of mouse cDNA clones to identify potential caspase-8 and -10 substrates. To identify novel substrates of caspase-8 and -10, we first *in vitro* transcribed and translated cDNA pools prepared from a mouse liver cDNA library in the presence of ^{35}S-methionine. The ^{35}S-labeled protein pool contained an average of more than 50 different proteins (data not shown). More than 1,500 different ^{35}S-labeled proteins, produced from 30 mouse liver cDNA pools, were examined *in vitro* for their proteolytic cleavage by caspase-8 and -10 (data not shown, and Fig. 1). When a protein pool containing a potential caspase-8 or -10 substrate was identified (for example, cDNA pool #6 in Fig. 1), the corresponding cDNA pool was progressively subdivided and re-examined in the same manner until a single positive cDNA clone was isolated (6,10). From the 30 mouse liver cDNA pools, we identified a total of six different genes encoding putative caspase-8 or -10 substrates (Table I). cDNAs encoding ABCF1 (ATP-binding cassette, subfamily F1), AKAP1 (A kinase anchor protein 1), CPE (carboxypeptidase E), DOPEY1 (dopey family member 1), and GOPC1 (golgi associated PDZ and coiled-coil motif containing transcription variant 1) were identified as truncated clones, and BTF3 (basic transcription factor 3) was isolated as a full-length cDNA (Tables I and II). The isolation of partial cDNAs did not affect either the screening or identification of positive clones in our caspase substrate screening system.

Verification of potential caspase-8 and -10 substrates. We next verified that the clones we had isolated in our screen for caspase-8 and -10 substrates represent *bona fide* substrates *in vitro*. We carried out the *in vitro* proteolytic cleavage assay with 10 units of active human caspase-8 or -10 and ^{35}S-labeled proteins *in vitro* transcribed and translated from the isolated mouse cDNA clones of ABCF1, AKAP1, BTF3, CPE, DOPEY1, and GOPC1 as substrates. Cleavage by caspase-8 of each of the six proteins, with the exception of ABCF1, was confirmed (Table II and Fig. 2). In contrast, caspase-10 recognized only ABCF1, AKAP1, and GOPC1 as substrates. Both caspase-8 and -10 completely cleaved AKAP1, whereas GOPC1 was more effectively cleaved by caspase-8 than by caspase-10. BTF3 and CPE were also efficiently cleaved by caspase-8. In the initial screen with pooled proteins, full-length BTF3 was completely cleaved by 10 units of caspase-8 (Fig. 1). However, when the same full-length BTF3 protein was synthesized from the isolated cDNA, it was not completely cleaved by 10 units of caspase-8 (Fig. 2). This observation is likely due to a difference in the amount of BTF3 protein present in each of the *in vitro* caspase cleavage assays. It is well established that the caspase proteolytic signaling cascades are interconnected and have overlapping substrate specificity (5,9). We do not exclude the possibility that these newly identified substrates could also be cleavage targets of other caspases, including caspase-3.

Table I. Isolation of mouse cDNA clones representing putative substrates of caspase-8 and -10.

Gene symbol	Isolated cDNA clone from mouse cDNA library		Full-length information (predicted) in the public sequence databases		
	ORF (bp)	Amino acid	ORF (bp)	Amino acid	Protein size (kDa)
ABCF1	2352	784	2619	873	95
AKAP1	1218	406	2571	857	92
BTF3	612	204	612	204	22
CPE	900	300	1428	476	53
DOPEY1	1335	445	3801	1267	141
GOPC1	936	312	1389	463	51

Table II. Cleavage of putative substrates by caspase-8 and -10.

Mouse gene		Cleavage by	
Symbol	Full name	Caspase-8	Caspase-10
ABCF1	ATP-binding cassette, subfamily F1	-	+
AKAP1	A kinase anchor protein 1	+++	+++
BTF3	Basic transcription factor 3	++	-
CPE	Carboxypeptidase E	++	-
DOPEY1	Dopey family member 1	+	-
GOPC1	Golgi associated PDZ and coiled-coil motif containing transcription variant 1	++	+

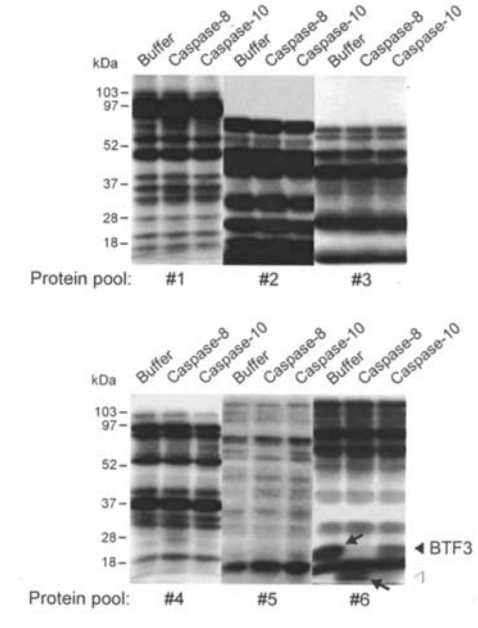

Figure 1. Screening the mouse liver cDNA library for putative substrates of caspase-8 and -10 *in vitro*. Pools of a mouse liver cDNA library were transcribed and translated in the presence of ^{35}S-methionine for 1 h at 30˚C *in vitro*. Ten microlitres of each of the ^{35}S-labeled protein pools (#1-6), containing an average of more than 50 different proteins, was then screened for cleavage by 10 units of recombinant and caspase-8 and -10 for 1 h at 37˚C. A positive protein pool, pool #6, was progressively subdivided and re-examined in the same manner until a single positive cDNA clone (in this case, BTF3) was isolated. Black arrows, protein bands of either uncleaved or cleaved BTF3; black arrowhead, uncleaved form of BTF3; white arrowhead, cleaved form of BTF3.

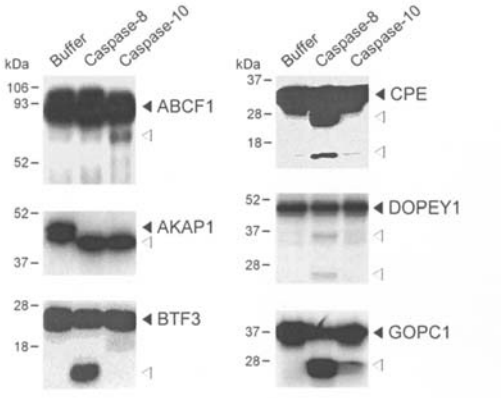

Figure 2. Identification of ABCF1, AKAP1, BTF3, CPE, DOPEY1, and GOPC1 as putative substrates of caspase-8 and -10 *in vitro*. A total of six positive cDNA clones were successfully isolated from a mouse liver cDNA library using the screening methodology described in Materials and methods. Ten microlitres of ^{35}S-labeled ABCF1, AKAP1, BTF3, CPE, DOPEY1, or GOPC1 produced from each isolated cDNA clone was used in the *in vitro* proteolytic cleavage assay with 10 units of caspase-8 or -10 for 1 h at 37˚C. Black arrow head, uncleaved form; white arrow head, cleaved form.

In silico analysis of putative substrates of caspase-8 and -10 (Table III). We next performed *in silico* analysis of the newly identified substrates of caspase-8 and -10 in order to pursue their potential involvement in apoptotic signaling networks. ABCF1 (also known as GCN20, ABC27 or

Table III. *In silico* analysis of putative caspase substrates identified from the mouse cDNA library screening system.

Human gene	Gene ID	Amino acid (size in kDa)	Chromosomal location	Subcellular localization	Amino acid		Interacting protein	Known or predicted molecular function and biochemical process
					Domains	Predicted motifs		
ABCF1	23	238 (27)	6p21.33	Expressed ubiquitously	ABC transporter (95-208)	Phosphorylation by PKC (T40)	EIF6	ATP-binding; responsible for the transport of various molecules across membranes; regulated by TNF-α; enhances protein synthesis and inflammation process
AKAP1	8165	903 (97)	17q21-q23	Mitochondria	Signal peptide (3-25) PKA binding (344-357), Ser/Thr-rich (489-540) KH (612-659), Tudor (756-815)	MAPK1 binding (L131), phosphorylation by ABL1 (Y684), GRB2 (P422, P534, P694) and PKA (631)	AAT1, CAV1, MYCBP, PPP1CA, PRKR1A, PRKAR1B, PRLAR2A, PTPN21	Binds to type I and II regulatory subunits of PKA and anchors them to mitochondria; involved in the cAMP-dependent recruitment of mRNA to a specific cellular compartment
BTF3	689	208 (22)	5q13.2	Nucleus	NAC (83-144)	Phosphorylation by ATM (S30) and PKC (T69)	CSNK2B, MED21, POLR2B	Forms a stable complex with RNA polymerase II; required for the initiation of transcription; phosphorylated upon DNA damage; cleaved by caspases during apoptosis
CPE	1363	572 (64)	4q32.3	Expressed ubiquitously, nucleus, peripheral membrane	Signal peptide (1-25), TM (102-124), Zn peptidase (271-561)	Phosphorylation by PKA (S94)	BDNF, CCK, GAST, GCG, INS, NTS, PMCH, TRH	Cleaves C-terminal amino acid residues; involved in the biosynthesis of peptide hormones and neurotransmitters
DOPEY1	23033	2369 (267)	6q15	Maybe plasma membrane	Dopey (2-307)	Not known	Not known	Not known
GOPC1	57120	462 (51)	6q21	Golgi apparatus, cytoplasm, peripheral membrane	PDZ (297-371), coiled-coil 82-123, 154-202)	Phosphorylation by PLCG1 (Y369)	Becn1, CFTR, Grid2, FZD5, RHOQ, STX6	Plays a role in intracellular protein trafficking and degradation; involved in the transport of chloride ions

ABC, ATP-binding cassette; EIF6, eukaryotic translation initiation factor 6; TNF, tumor necrosis factor; PKA, protein kinase A; KH, K-homology; MAPK1, mitogen-activated protein kinase 1; ABL1, v-abl Abelson murine leukemia viral oncogene homolog 1; GRB2, growth factor receptor-bound protein 2; AAT1, AMY1-associated protein expressed in testis 1; MYCBP, c-myc binding protein AMY-1; CAV1, caveolin 1; PP1CA, protein phosphatase 1 catalytic subunit α; PRKAR1A, PKA regulatory subunit type I α; PRKAR1B, PKA regulatory subunit type I ß; PRKAR2A, PKA regulatory subunit type II α; PTPN21, protein tyrosine phosphatase non-receptor type 21; NAC, nascent polypeptide-associated complex; CSNK2B, casein kinase 2 ß subunit; MED21, mediator complex subunit 21; POLR2B, RNA polymerase II polypeptide B; BDNF, brain-derived neurotrophic factor; CCK, cholecystokinins; GAST, gastrin; GCG, glucagons; INS, insulin; NTS, neurotensin; PMCH, pro-melanin concentrating hormone; TRH, thyroliberin; PDZ, PSD-95, Dlg, and ZO-1/2; PLCG1, phospholipase C γ 1; CFTR, cystic fibrosis transmembrane conductance regulator; FZD5, frizzled-5; RHOQ, Rho-related GTP-binding protein RhoQ; and STX6, syntaxin-6.

ABC50) is a member of the superfamily of ATP-binding cassette (ABC) transporters which are responsible for the transport of a variety of molecules across membranes (13). Unlike other members of the superfamily, ABCF1 lacks the transmembrane domain that is characteristic of ABC transporters. It is likely that ABCF1 is regulated by tumor necrosis factor-α (TNF-α) and plays a role in the enhancement of protein synthesis and the inflammation process (13). Caspase-10 can cleave ABCF1 *in vitro* (Table II and Fig. 2). This suggests that in apoptotic cells, ABCF1 may be a target for caspase-10 and also possibly other caspases.

AKAP1 (also known as AKAP149) is a member of the AKAP family, a group of structurally diverse proteins that bind to the regulatory subunits of PKA and confine them to discrete locations within the cell (14). AKAP1 localizes to the outer membrane of mitochondria through its N-terminal signal peptide (amino acids 3-25), and tethers PKA to the mitochondrial outer surface (15). Phosphorylation of specific sites in the K-homology (KH) domain of AKAP1 by PKA stimulates its binding to the 3'-untranslated region of the mRNAs of manganese superoxide dismutase and Fo-ATP synthase. This promotes the translocation of these mRNAs from the cytosol to the mitochondria (16). These data suggest that AKAP1 is involved in cAMP-dependent intracellular signaling pathways that regulate cell survival. Our data indicate that AKAP1 can be cleaved by both caspase-8 and -10 (Table II and Fig. 2). Activation of these initiator caspases during apoptosis could result in the cleavage of AKAP1, potentially releasing its regulatory targets and thereby stimulating cell death at an early step in the apoptotic signaling pathways. We are currently examining this possibility.

It has been reported that BTF3 (also known as NACB or ß-NAC) forms a stable complex with RNA polymerase IIB and is required for transcription initiation (17). Notably, it has also been shown that the *C. elegans* orthologue of BTF3, inhibitor of cell death-1 (ICD1), is necessary and sufficient to prevent apoptosis (18) and contains a putative caspase cleavage site. Furthermore, human BTF3 is a caspase-8 substrate that is rapidly eliminated from dying cells (19,20). These observations are consistent with our finding that BTF3 is a caspase-8-specific substrate *in vitro* (Table II and Fig. 2). This confirms that our *in vitro* systematic screening system is capable of finding *bona fide* caspase substrates.

CPE, a protease that cleaves C-terminal amino acid residues of proteins, is involved in neuropeptide processing and the biosynthesis of peptide hormones and neurotransmitters, including insulin (21-23). Human GOPC1 (also known as CAL, FIG or PIST) is a PDZ domain containing Golgi protein (24). It can interact with the Rho-related GTP-binding protein RhoQ (RNOQ) (25), syntaxin-6 (STX6) (26), frizzled-5 (FZD5) (27), cystic fibrosis transmembrane conductance regulator (CFTR) (28), Grid2 (29), and Becn1 (29). Based on these observations, it has been proposed that GOPC1 may play a role in intracellular protein trafficking and degradation and the regulation of chloride ion transport. It is possible that the biochemical activities of CPE and GOPC1 may be inactivated by caspase-8 and/or -10 during cell death.

In this study we constructed a mouse cDNA library and used it to systematically screen for novel substrates of caspase-8 and -10 *in vitro*. Using this methodology, we successfully identified five novel substrates that might be targeted specifically by the initiator caspases 8 and 10 in the early stages of apoptosis. These findings could provide important information on the mechanisms of apoptosis, in particular those mediated by death receptors.

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