The human homologue of the yeast polyubiquitination factor Ufd2p is cleaved by caspase 6 and granzyme B during apoptosis

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In the present study, we demonstrate that a human homologue of Ufd2p (a yeast protein that catalyses the formation of long polyubiquitin chains, and is implicated in responses to environmental stress), UFD2 (ubiquitin fusion degradation protein-2), is cleaved during apoptosis induced by multiple stimuli, including UVB irradiation, Fas ligation, staurosporine treatment and cytotoxic lymphocyte granule-induced death. Caspase 6 and granzyme B efficiently cleave UFD2 [$k_{cat}/K_m = (4–5) \times 10^4$ $M^{-1} \cdot s^{-1}$] at Asp^{123}, whereas caspases 3 and 7 cleave UFD2 approx. 10-fold less efficiently immediately upstream at Asp^{109}. Thus UFD2 is added to the growing list of proteins with closely spaced caspase and granzyme B cleavage sites, suggesting the presence of a previously unrecognized, conserved motif. Both cleavage sites are contained and conserved within a novel 300-amino-acid N-terminal domain present in apparent UFD2 orthologues in mice and zebrafish, but absent in all UFD2 family members in lower eukaryotes. Full-length recombinant UFD2 exhibited ubiquitin–protein ligase ('E3')-like ubiquitination activity *in vitro*, but this activity was abolished in recombinant UFD2 truncated at the granzyme B/caspase 6 cleavage site. Cleavage of UFD2 by caspases or granzyme B within this putative regulatory N-terminal domain might have important functional consequences within the apoptotic cascade.

Key words: autoantigen, granule pathway, proteasome, U box, ubiquitin.

INTRODUCTION

In recent years it has become evident that programmed cell death, or apoptosis, has a critical role in many aspects of human biology. The importance of an intact apoptotic pathway can clearly be seen in studies where defects in the apoptotic machinery lead to impaired development, increased risk of neoplasms and autoimmune disease [1–3]. The key effector molecules in the apoptotic cascade are caspases, a group of cysteine proteases expressed ubiquitously that cleave specific protein substrates following aspartate residues, within the context of a tetrapeptide recognition sequence [4]. When an upstream caspase is activated, it initiates a proteolytic cascade in which downstream caspases cleave a variety of nuclear and cytoplasmic protein substrates, ultimately leading to cell death. The identity and regulation of these caspase substrates has been the subject of intense research.

While all cells appear to be capable of inducing within themselves an apoptotic death programme, cytotoxic T lymphocytes and natural killer cells can also trigger apoptosis in an adjacent target cell. Granules containing the pore-forming protein perforin, and serine proteases called granzymes, are released from the attacking cell, and are internalized into target cells by cation-independent mannose-6-phosphate-receptor-dependent endocytosis [5]. Granzymes induce apoptosis in the target cell by cleaving pro-caspases into their corresponding active forms [6], and also by direct caspase-independent cleavage of a variety of important cellular substrates [7,8], especially Bid [9–11]. The serine protease granzyme B is structurally and mechanistically unrelated to the cysteine-active caspases, but shares with caspases a near-absolute requirement for aspartate in the P_1 position of substrate proteins. Interestingly, many caspase substrates are also directly cleaved by granzyme B, but at unique sites [7,12–14], reflecting the distinct tetrapeptide specificity of this serine protease [15,16].

Many caspase and granzyme B substrates are autoantigens targeted in systemic autoimmune disease [14,17], making autoantibodies useful probes to identify molecules cleaved during apoptotic death. Using a patient's serum recognizing an unidentified 130 kDa autoantigen cleaved during apoptosis, we report here the cloning and identification of the human autoantigen ubiquitin fusion degradation protein-2 (UFD2), a homologue of the yeast protein Ufd2p. Ubiquitin chain formation is an ATP-dependent process in which a ubiquitin–protein ligase (E3) transfers free ubiquitin on to a polypeptide in the presence of a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2). Yeast Ufd2p was recently identified as an additional factor (E4) required for formation of long polyubiquitin chains, thus allowing the targeting of ubiquitinated substrates for destruction by the 26 S proteasome [18]. We show that human UFD2 is potently cleaved by both caspases and granzyme B during apoptosis, within a novel N-terminal extension absent in other UFD2 family members, but highly conserved among

Abbreviations used: DEVD-CHO, acetyl-Asp-Glu-Val-Asp aldehyde; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin–protein ligase; EST, expressed sequence tag; IVTT, *in vitro* transcription/translation; LAK, lymphokine-activated killer; PARP, poly(ADP-ribose) polymerase; RT, reverse transcriptase; UFD2, ubiquitin fusion degradation protein-2.

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apparent vertebrate orthologues. Interestingly, a UFD2 fragment corresponding to cleavage at the caspase 6/granzyme B cleavage site exhibits markedly reduced polyubiquitination activity *in vitro*. Cleavage of UFD2 within this putative regulatory N-terminal domain might have important functional consequences within the apoptotic cascade.

EXPERIMENTAL

Screening of autoimmune sera

Autoimmune patients' sera were used to probe Western blots of detergent lysates from control and apoptotic HeLa cells and neutrophils. Apoptosis was induced in HeLa cells by UVB irradiation [17], and in neutrophils by overnight culture in tissue-culture media [19]. HeLa cells were lysed in Buffer A containing 1 % (v/v) Nonidet P40, 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 20 μM chymostatin, 2 μg/ml each of antipain and pepstatin, 5 μg/ml leupeptin and 1 mM PMSF. Neutrophils were lysed by nitrogen cavitation, as described previously [19]. Lysates (80 μg of protein) were resolved by SDS/PAGE (10 % gels), and transferred on to nitrocellulose. The membranes were blocked with 3 % (w/v) BSA and then probed with patients' sera diluted 1:5000, followed by detection using horseradish-peroxidase-labelled goat anti-(human IgG) (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) and enhanced chemiluminescence.

Protease assays

To assay cleavage of endogenous protease substrates in cell lysates, HeLa cells were lysed in Buffer B containing 10 mM Hepes/KOH, pH 7.4, 2 mM EDTA, 5 mM dithiothreitol and 1 % (v/v) Nonidet P40, plus 2 μg/ml pepstatin, 5 μg/ml leupeptin and 2 mM PMSF. Lysates were incubated with purified human granzyme B [20] or recombinant caspases 3, 6, 7, 8 and 9 (gifts from Nancy Thornberry at Merck Research Labs, Rahway, NJ, U.S.A.) at 37 °C for 60 min. In certain of the experiments, 5 mM iodoacetamide was added to inhibit endogenous caspases. Reactions were stopped by addition of SDS/PAGE sample buffer, and intact substrates and their cleaved products were visualized by Western blotting.

Expression cloning of UFD2

Plaque lifts from a commercially prepared HeLa cell cDNA λ phage library (Clontech, Palo Alto, CA, U.S.A.) were probed with patient serum 61517 (1:2500), followed by ^{125}I-labelled Protein A. Positives were re-screened until a single plaque-pure clone was obtained. The clone was sequenced in its entirety by standard methods.

A full-length human UFD2 clone was generated by reverse transcriptase (RT)-PCR from HeLa cell total RNA using primers containing restriction sites present in the authentic UFD2 sequence: TTGGTCTCGAGAACAGAAGGATCTCTCC (5′, *Xho*I site underlined) and CCCTTAAGCTTTTGACCGTGT-TTGTCAC (3′, *Hin*dIII site underlined). The resulting PCR product was gel-purified, ligated into the T/A cloning vector pCR 2.1 (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions, and sequence-verified.

Northern blotting

Human normal tissue and human cancer-cell-line Northern blots (Clontech) were probed with a random primer labelled 401 bp fragment of UFD2, according to the manufacturer's instructions.

Immunoprecipitation of recombinant human UFD2

[^{35}S]Methionine-labelled recombinant UFD2 was made using coupled *in vitro* transcription/translation (IVTT; Promega). Aliquots were diluted in 500 μl of Buffer A supplemented with 0.2 % (w/v) BSA, pre-cleared with Protein A–agarose beads, and then agitated for 30 min at 4 °C with 1 μl of patient serum 61517 or normal human serum. After the addition of Protein A–agarose beads, the immunoprecipitated proteins were washed and then released from the beads by boiling in SDS/PAGE sample buffer. The samples were resolved on SDS/PAGE (8 % gels) and analysed by fluorography.

In vitro cleavage of recombinant human UFD2

[^{35}S]Methionine-labelled recombinant UFD2, prepared using IVTT, was diluted with Buffer B and incubated with recombinant human caspases or purified human granzyme B in a final reaction volume of 25 μl. After incubation for 60 min at 37 °C, reactions were terminated by addition of SDS/PAGE sample buffer, and analysed as described above. Values for k_{cat}/K_m were estimated from densitometric scans of films based on the first-order rate equation:

$$\% \text{ Cleaved} = 100 \times \left\{ 1 - e^{-\left(\frac{k_{cat}}{K_m} \cdot [E] t \right)} \right\}$$

where [E] represents the concentration of enzyme and t is the time. Note that protease concentrations were measured by protein assay. Since active-site titration [21] to determine the proportion of fully active enzyme was not performed, 'true' k_{cat}/K_m values might be higher than those indicated here.

Site-directed mutagenesis

Point mutations were introduced to convert aspartate codons into alanine codons using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Mutations were confirmed by sequence analysis.

Induction of apoptosis

HeLa cells were incubated in the presence of 1 μM staurosporine (Sigma, St Louis, MO, U.S.A.) for 4 h, or were UVB-irradiated followed by incubation in culture for 6 h. Jurkat cells were incubated in the absence or presence of 1 μg/ml CH-11 anti-Fas monoclonal antibody (MBL International, Watertown, MA, U.S.A.) for 2 h [22]. Assays of cytotoxic granule-induced cleavage were performed by incubating purified human lymphokine-activated killer (LAK) cells with target K562 erythroblast cells for 4 h with an effector:target ratio of 3:1, as described previously [7], in the presence or absence of the caspase 3/7 inhibitor acetyl-Asp-Glu-Val-Asp aldehyde (DEVD-CHO; Calbiochem, La Jolla, CA, U.S.A.) at a concentration of 100 μM.

Production and purification of recombinant UFD2 from baculovirus

Full-length or C-terminal (truncated length, lacking amino acid residues 2–123 corresponding to the granzyme B/caspase 6 cleavage site) UFD2 sequences containing C-terminal histidine tags were generated by PCR and inserted into pBacPAK8 (Clontech). Recombinant viruses were generated and used to infect Sf21 cells. Infected cells were lysed, and recombinant proteins were purified by Ni^{2+}-nitrilotriacetate chromatography. Eluted recombinant proteins (> 95 % pure, as judged by SDS/PAGE) were dialysed against 20 mM Tris/HCl, pH 7.5, containing 10 % (v/v) glycerol, and stored at −80 °C in small aliquots.

In vitro E3 assay

E3-like ubiquitination activity was assayed by the method of Hatakeyama and co-workers [23]. Purified histidine-tagged full-length or C-terminal UFD2 (1.5 μg) was added to a 30 μl reaction mixture comprising 20 mM ubiquitin (Sigma), 50 mM Tris/HCl, pH 7.5, 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM ATP, 1 mM creatine phosphate, 0.5 unit of creatine phosphokinase and 2 μl of *Escherichia coli* extract, with or without 100 nM purified rabbit E1 (Boston Biochem., Boston, MA, U.S.A.) and 2 μg of the recombinant E2 enzyme glutathione S-transferase–UbcH5c (Boston Biochem). Since ubiquitination does not occur in prokaryotes, the bacterial lysate provides a source of exogenous ubiquitin acceptor substrate, without introducing interfering ubiquitination enzymes. The reaction was incubated for 2 h at 30 °C, and stopped by the addition of SDS/PAGE sample buffer. The reaction products were analysed by SDS/PAGE and Western blotting, using a monoclonal antibody that specifically recognizes multi-ubiquitin chains (clone FK2; MBL International).

RESULTS

Patient serum 61517 recognizes a 130 kDa antigen that is cleaved by caspases and granzyme B

Screening sera from patients with systemic autoimmune disease by immunoblotting has allowed us to characterize a large number of autoantigens that are cleaved by aspartic-acid-specific proteases during apoptosis [24]. The serum from one such patient recognized a 130 kDa band in control cell lysates (Figure 1A). In lysates made from apoptotic HeLa cells or neutrophils, a 110 kDa band was also immunoblotted by this serum (results not shown). When lysates of control HeLa cells were treated with purified human granzyme B (in the presence of iodoacetamide to inhibit activation of endogenous pro-caspases) or caspases 3 or 6, similar fragments were observed. The fragment produced by caspase 3 migrated slightly more slowly on SDS/PAGE gels (see below). The autoantigen recognized by serum 61517 is therefore cleaved during apoptosis in several cell types, and is similarly cleaved in control cell lysates by addition of either caspases or granzyme B.

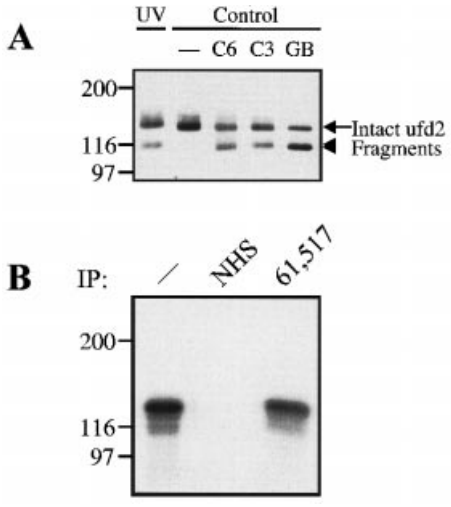

Figure 1 Patient serum 61517 recognizes human UFD2, an autoantigen that is cleaved by several different aspartic acid-specific proteases

(**A**) Control HeLa cells and HeLa cells treated with UVB to induce apoptosis were lysed in Buffer B and treated with recombinant human caspases or granzyme B. Lysates were analysed by Western blotting with patient 61517 serum. UV, lysate prepared from HeLa cells induced to become apoptotic by UVB irradiation; —, HeLa cell lysate with no additions; C6, HeLa lysate treated with caspase 6; C3, HeLa lysate treated with caspase 3; GB, HeLa lysate treated with granzyme B and 5 mM iodoacetamide, to inhibit endogenous caspases. (**B**) [^{35}S]Methionine-labelled recombinant UFD2 was resolved by SDS/PAGE either directly (—), or after immunoprecipitation (IP) with normal human serum (NHS) or patient serum 61517. Migration of molecular-mass markers (in kDa) is indicated on the left.

The autoantigen recognized by patient serum 61517 is human UFD2

To identify this autoantigen, patient serum 61517 was used to screen a HeLa cell λ-phage expression library. After several rounds of purification, a single positive 1089 bp clone was obtained. Sequence analysis showed that this clone represented a fragment of the human homologue of the *Saccharomyces cerevisiae UFD2* gene. A search of GenBank® revealed a matching sequence from human fetal brain (accession no. AF043117), determined as part of a large-scale sequencing project around chromosome 1p36. Using primers based on the human fetal brain sequence, a full-length version of human UFD2 was generated by RT-PCR from HeLa cell cDNA. In order to confirm that human UFD2 is the autoantigen recognized by patient serum 61517, radiolabelled recombinant UFD2 was synthesized by IVTT. The product: (i) migrated to the expected size on SDS/PAGE; (ii) was immunoprecipitated by patient serum 61517, but not by control human serum (Figure 1B); and (iii) generated a 110 kDa fragment after cleavage by caspases and granzyme B (see below).

Sequence analysis of the RT-PCR product (Figure 2A) showed an open reading frame encoding 1173 amino acids, identical with the human fetal brain sequence in the database except for a 387 bp deletion representing a 129-amino-acid in-frame truncation, 270 amino acids from the N-terminus. This difference seems likely to represent an alternative splicing event. Indeed, a partial genomic DNA entry in the High Throughput Genome Sequencing database (accession no. AL096841) shows an apparent intron sequence inserted at that position of the cDNA sequence, flanked by canonical splice donor and acceptor sites. Despite the fact that two different sized bands were detected by Northern blotting (see below), attempts to amplify a PCR fragment across the missing exon from HeLa cell cDNA resulted in only the shorter fragment corresponding to the sequence in Figure 1, indicating that the shorter splice form is the predominant species expressed in HeLa cells. The nature of the two bands detected by Northern blotting is unknown.

Several UFD2-like protein sequences are present in the public database (Figure 2B), representing a broad range of eukaryotic organisms. The highest degree of similarity exists in an approx. 70-amino-acid region at the extreme C-termini of these sequences. This domain, which has been called the 'U box' [18], is presumed to be critical for the activity of these proteins. Amino acid identity among all sequences shown is 51–60 % within the U box. The remainder of human UFD2 is approx. 25–30 % identical with the other proteins shown here, with the exception of an approx. 300-amino-acid N-terminal extension, which has no significant similarity to the other UFD2-like proteins, or to any other proteins in the database (see below). In addition to human UFD2, a second human protein, called KIAA0126, contains an apparent U box. UFD2 and KIAA0126 are 60 % identical within the U box, and 26 % identical overall. It is unknown whether these two proteins are related functionally. Two sequences, from mouse and zebrafish [partial sequence, as deduced from an expressed sequence tag (EST)], show a significantly greater degree

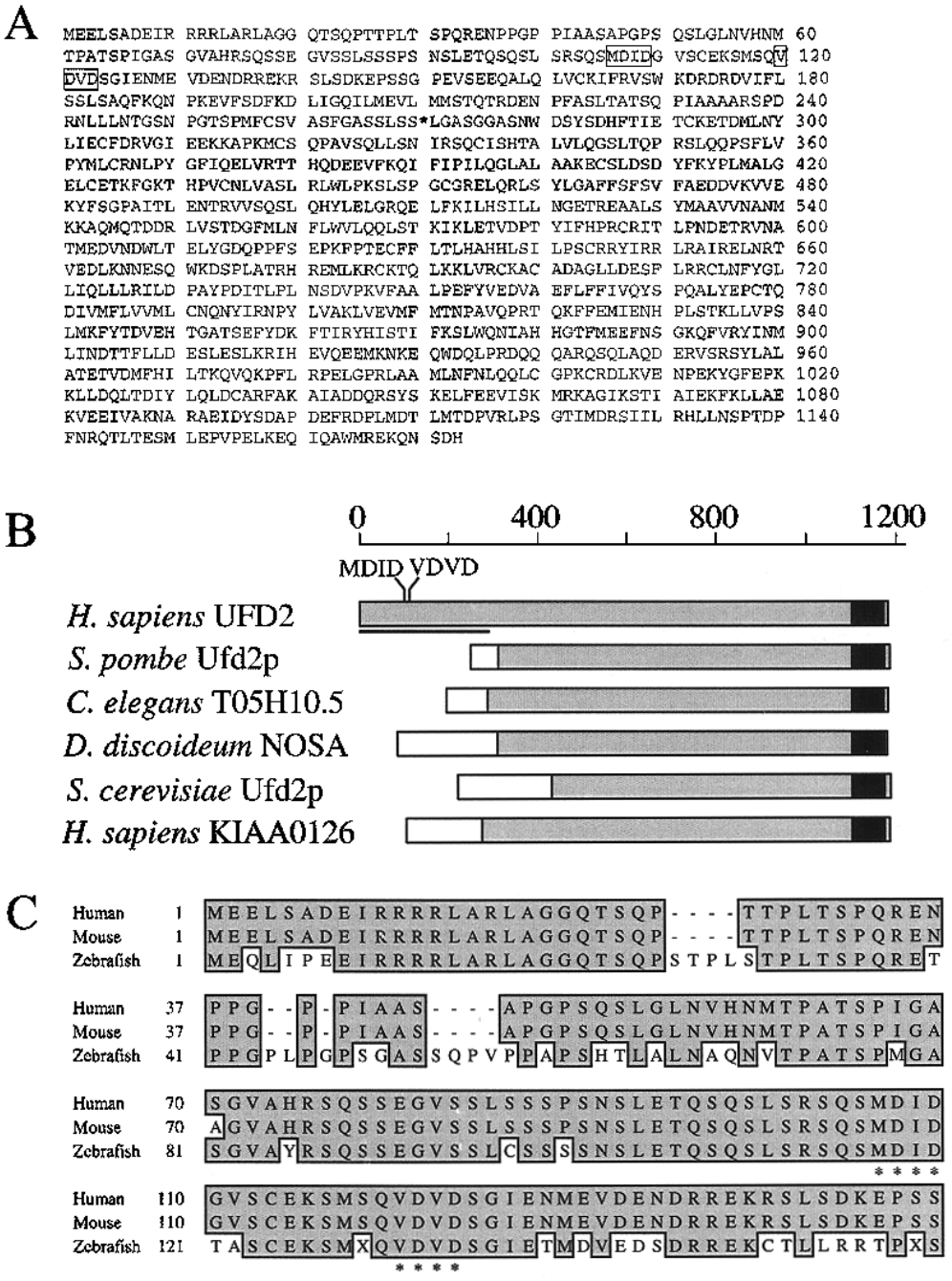

Figure 2 Sequence analysis of human UFD2

(**A**) Deduced amino acid sequence of the human cDNA for UFD2. The asterisk indicates the position of the putative alternatively spliced exon (see the text for further details). The two tetrapeptide recognition sequences for caspases and granzyme B (see below) are boxed. The cDNA and deduced amino acid sequence have been deposited in the GenBank® database (accession number AF331520). (**B**) Domain comparison of human UFD2 and related proteins. Human UFD2 is shown as a grey bar, as are the similar regions of the other proteins. Regions of the other proteins with no apparent similarity to human UFD2 are shown as open bars. The U box domain of each protein is shown as a black bar. The human UFD2 novel amino terminal extension is indicated with a heavy underline. The positions of the tetrapeptide recognition sequences are indicated. Amino acid numbers (for human UFD2) are shown at top. The accession numbers for the sequences shown are as follows: *Homo sapiens* UFD2, AAK69622; *Schizosaccharomyces pombe* Ufd2p, AAC80427; *Caenorhabditis elegans* T05H10.5, Q09349; *D. discoideum* NOSA, AAC34746; *Saccharomyces cerevisiae* Ufd2p, AAC49024; *H. sapiens* KIAA0126, Q14139. (**C**) ClustalW alignment of the N-terminal portion of human UFD2 with two putative vertebrate species orthologues, as deduced from the EST database. Residues identical with the human sequence are enclosed in grey-shaded boxes. Asterisks indicate the positions of the tetrapeptide recognition sequences. The accession numbers for the sequences shown are as follows: mouse, AAG17287; zebrafish EST, AI657882.

of similarity to human UFD2. With the exception of the more distantly related KIAA0126, these represent the only other vertebrate sequences in this group, and probably represent species orthologues of human UFD2. The mouse and zebrafish sequences, which are approx. 97% and 90% identical with human UFD2 over all regions of the molecules present in the

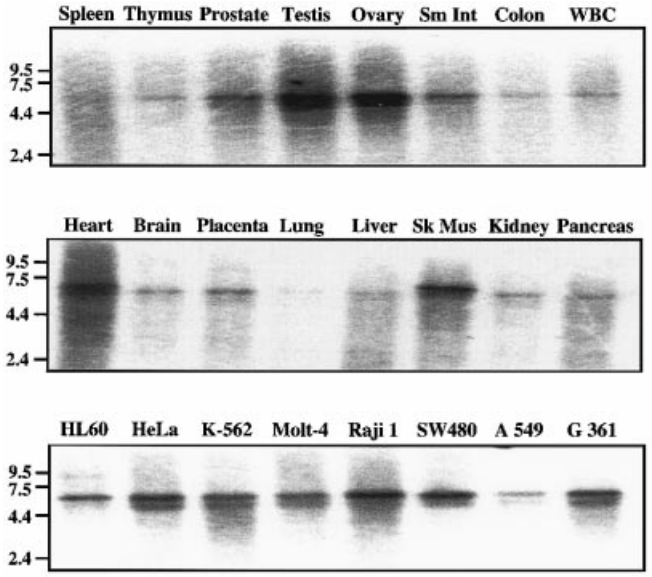

Figure 3 Northern blot analysis of human UFD2

Human tissue and cell line Northern blots were probed with a ^{32}P-labelled fragment of human UFD2. Sm Int, small intestine; WBC, white blood cells; Sk Mus, skeletal muscle. RNA size markers are indicated at the left (in kb).

database, are the only members of this group that contain the N-terminal extension present in human UFD2 (Figure 2C).

Expression of UFD2 in human tissues

The expression pattern of UFD2 in human tissues was examined by Northern blotting (Figure 3). RNA species of 5.5–6.5 kb were detected to differing degrees in a wide variety of tissues. In some tissues, two closely spaced bands were evident, probably representing alternatively spliced transcripts. The highest level of expression was seen in the ovary, testis, heart and skeletal muscle. Expression was low in the colon, thymus and peripheral blood leukocytes, and almost undetectable in the lung and spleen. Among a cohort of human cancer cell lines, UFD2 mRNA was relatively abundantly expressed in all except A549 (lung carcinoma) cells.

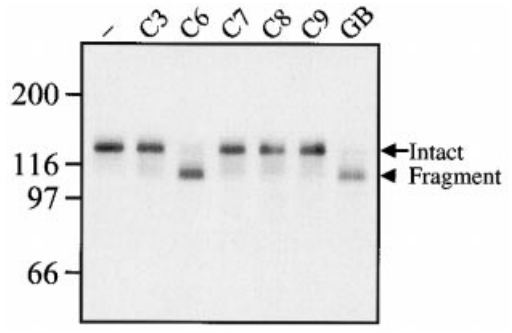

Figure 4 Recombinant UFD2 is cleaved by caspases and granzyme B

[^{35}S]Methionine-labelled recombinant UFD2 was incubated for 1 h at 37 °C either alone or with recombinant human caspases [caspase 3 (C3), C6, C7, C8 and C9] or purified human granzyme B (GB) at a final concentration of 20 nM. Reactions were analysed by SDS/PAGE and fluorography. Migration of molecular-mass markers (in kDa) is indicated at left.

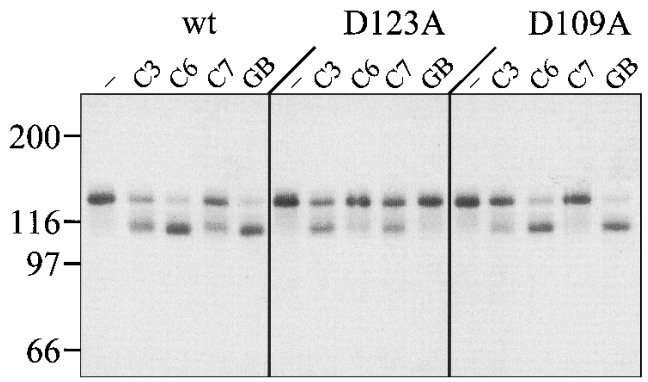

Figure 5 Identification of caspase and granzyme B cleavage sites by site-directed mutagenesis: caspase 6 and granzyme B share a common cleavage site

Wild-type (WT) UFD2, and UFD2 bearing Asp^{123}Ala (D123A) or Asp^{109}Ala (D109A) mutations, were labelled with [^{35}S]methionine and then incubated with recombinant caspases 3 (C3; 100 nM), 6 (C6; 20 nM) or 7 (C7; 100 nM), or with purified granzyme B (GB; 20 nM). Reactions were analysed by SDS/PAGE and fluorography. Migration of molecular-mass markers (in kDa) is indicated at the left.

Table 1 Estimated k_{cat}/K_m values of selected proteases for cleavage of recombinant human UFD2

[^{35}S]Methionine-labelled UFD2 was incubated *in vitro* with various concentrations of the indicated proteases. The data from the resulting densitometric scans were used to calculate k_{cat}/K_m values on the basis of the reduction in intensity of intact UFD2, as described in the Experimental section. Enzyme concentrations with residual intact UFD2 of between 85 and 15% of the control (no enzyme) were used in the calculations. The mean of the values (2–4 data points) from each independent experiment represents an *n* of 1, and experiments were pooled to determine the mean ± S.D. shown.

Protease	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	*n*
Caspase 3	$4.8 \pm 0.3 \times 10^3$	4
Caspase 6	$4.0 \pm 0.7 \times 10^4$	3
Caspase 7	$3.4 \pm 0.8 \times 10^3$	2
Granzyme B	$5.0 \pm 2.3 \times 10^4$	3

Cleavage of recombinant UFD2

Radiolabelled recombinant UFD2 was cleaved potently by both caspase-6 and granzyme B (Figure 4), generating a 110 kDa product that co-migrated with that observed for endogenous UFD2 in apoptotic HeLa cells (results not shown). Less efficient cleavage was observed with caspases 3 and 7 (not apparent at this enzyme concentration; see Figure 5 below), and caspases 8 and 9 had no significant effect. The fragment generated by caspase 6 and granzyme B migrated slightly more rapidly on SDS/PAGE than that generated by the other caspases (Figure 5, first panel). The susceptibility of recombinant UFD2 to cleavage by a cohort of caspases and granzyme B was quantified by performing *in vitro* cleavage assays over a range of caspase and granzyme B concentrations, and calculating k_{cat}/K_m values for each protease (Table 1). The k_{cat}/K_m values for caspase 6 and granzyme B [approx. $(4–5) \times 10^4$ $M^{-1} \cdot s^{-1}$] were approx. 10-fold higher than those for caspases 3 and 7 [approx. $(3–5) \times 10^3$ $M^{-1} \cdot s^{-1}$]. The efficiency of UFD2 cleavage by granzyme B compares

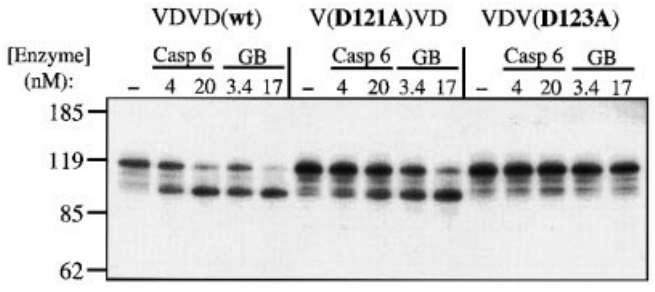

Figure 6 The tetrapeptide site preferences of caspase 6 and granzyme B are different

[^{35}S]Methionine-labelled recombinant UFD2, either wild-type (wt) or containing an Asp → Ala mutation at amino acid position 121 or 123, was incubated with increasing concentrations of recombinant caspase 6 (Casp 6) or purified granzyme B (GB). Reactions were analysed by SDS/PAGE and fluorography. Migration of molecular-mass markers is indicated at the left (in kDa). Note that the Asp^{121}Ala mutant is efficiently cleaved by granzyme B, but not by caspase 6. Neither enzyme cleaves the Asp^{121}Ala mutant.

favourably with the efficiency of granzyme B cleavage of pro-caspase 3 determined previously in our laboratory ($k_{cat}/K_m = 3.6 \times 10^4$ $M^{-1} \cdot s^{-1}$ [7]). Note that Stennicke and co-workers [25] reported significantly higher k_{cat}/K_m values (approx. 5×10^6 $M^{-1} \cdot s^{-1}$) for granzyme B cleavage of pro-caspase 3, using caspase activity against a fluorescent substrate rather than pro-enzyme cleavage as an endpoint. This discrepancy might be a result of the considerably different assay system, or might result from a significant proportion of inactive enzyme in our preparation (see the Experimental section), or both.

Site-directed mutagenesis of UFD2

In order to understand more fully the biochemical nature of caspase- and granzyme B-mediated UFD2 cleavage, the primary sites of cleavage were mapped by site-directed mutagenesis and *in vitro* cleavage assays. Potential protease cleavage sites were identified on the basis of substrate specificity in combinatorial tetrapeptide libraries [15], then were confirmed or excluded by mutation of the putative P_1 aspartate to alanine, followed by cleavage of the IVTT-labelled recombinant substrates. Both caspase 6 and granzyme B cleaved UFD2 after Asp^{123} within the tetrapeptide Val-Asp-Val-Asp^{123} (VDVD^{123}; Figure 5), in good agreement with the expected cleavage specificities for these two proteases. Replacement of Asp^{123} with alanine caused complete abolition of 110 kDa fragment production by both proteases, and no new fragments were detected.

An analogous approach was used to investigate the cleavage sites of caspases 3 and 7 in UFD2. Whereas mutation of Asp^{123} had no effect on cleavage efficiency by these proteases, replacement of Asp^{109} with alanine within the tetrapeptide Met-Asp-Ile-Asp^{109} (MDID^{109}) blocked cleavage by both of these closely related caspases (Figure 5). The presence of this tetrapeptide 14 amino acids upstream from the caspase 6/granzyme B site agrees well with the slightly larger fragment seen after caspase 3/7 cleavage of endogenous UFD2 (Figure 1A, and results not shown) and recombinant UFD2 (Figure 5, left panel). Interestingly, the MDID tetrapeptide differs significantly from the sequence (DEVD) typically recognized by caspases 3 and 7. Normally, negatively charged residues are strongly favoured in the P_4 position [15]. This deviation from the optimal recognition sequence of these caspases might explain the relatively low k_{cat}/K_m values measured here.

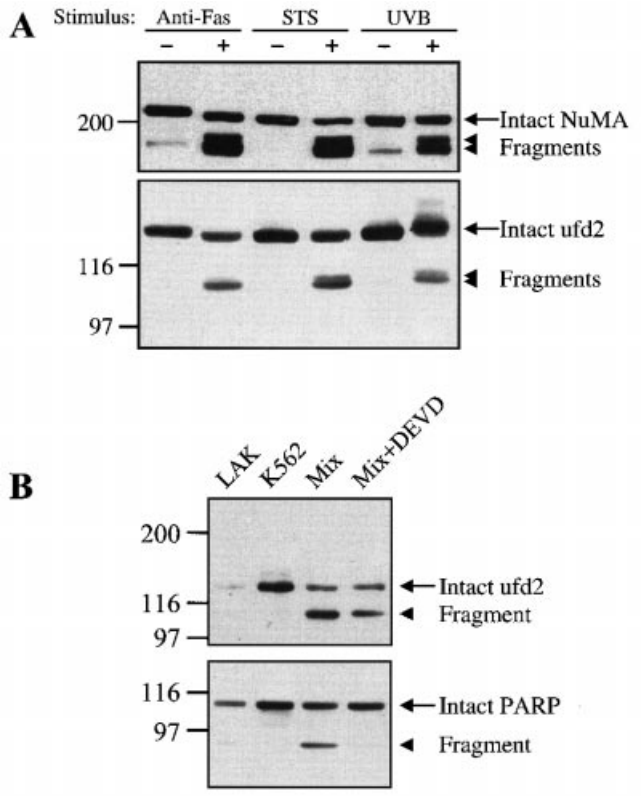

Figure 7 Multiple different apoptotic stimuli produce similar cleavage of UFD2 *in vivo*

(**A**) Biochemical apoptotic stimuli resulting in UFD2 cleavage. Jurkat cells were made apoptotic by Fas ligation (Anti-Fas), or HeLa cells made apoptotic by staurosporine (STS) treatment or UVB irradiation. Lysates were analysed by Western blotting using serum specific for nuclear mitotic apparatus protein (NuMA) [24] (upper panel) or serum 61 517 recognizing UFD2 (lower panel). (**B**) Cleavage of UFD2 during cytotoxic granule-induced apoptosis. Purified human LAK cells were incubated with K562 cells for 4 h, with or without the caspase 3/7 inhibitor DEVD-CHO (DEVD). The mixed cell population was collected, lysed and analysed by Western blotting using serum 61 517 recognizing UFD2 (upper panel) or serum recognizing PARP (lower panel). Migration of molecular-mass markers (in kDa) is indicated at left.

Since caspase 6 and granzyme B recognized the same tetrapeptide cleavage site in UFD2, we investigated further the fine structural specificity of these two evolutionarily unrelated proteases. Site-directed mutagenesis followed by *in vitro* protease assays showed that, while both proteases cleaved wild-type UFD2 at Asp^{123} with almost identical efficiency, mutation of the P_3 aspartate to alanine, i.e. V(D^{121} → A)VD, abolished caspase 6 proteolytic activity, whereas granzyme B activity was essentially unaffected (Figure 6). These data confirm that the relatively restricted specificity of the caspase family at the P_3 position [15,26] among peptide substrates is maintained for macromolecular substrates, and is not a feature of granzyme B.

Multiple apoptotic pathways lead to cleavage of UFD2

To test whether endogenous UFD2 cleavage was restricted to UVB-induced apoptosis, cells were given various stimuli to induce apoptosis via different pathways (Figure 7A). Treatment of HeLa cells with the protein kinase inhibitor staurosporine, and antibody-mediated ligation of Fas on Jurkat cells, both led to efficient cleavage of UFD2, with the same fragments detected as found after UVB irradiation-induced apoptosis. Cleavage of

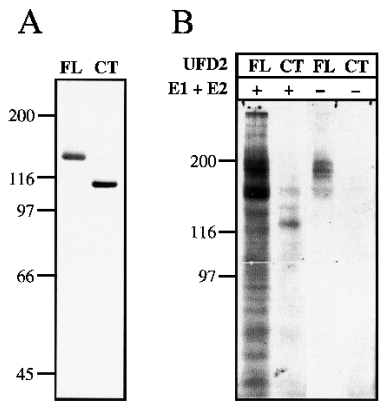

Figure 8 The domain removed by granzyme B/caspase 6 cleavage is required for polyubiquitination activity *in vitro*

(**A**) Coomassie Blue staining of recombinant UFD2 proteins expressed in baculovirus. Recombinant proteins (1.5 μg) were resolved by SDS/PAGE (8% gels) and stained with Coomassie Brilliant Blue. FL, full length; CT, C-terminal portion lacking amino acids 2–123. (**B**) E3-like polyubiquitination assay. Recombinant proteins were incubated for 2 h at 30 °C with ubiquitin, an ATP-regeneration system and *E. coli* protein lysate as a potential source of ubiquitin acceptor substrate, in the presence or absence of purified E1 and E2 proteins. Reactions were analysed by SDS/PAGE and Western blotting using a monoclonal antibody specifically recognizing multi-ubiquitin chains. Migration of molecular-mass markers is indicated at the left of both gels (in kDa).

the well-described caspase substrate, nuclear mitotic apparatus protein, was assayed in parallel as a positive control. Interestingly, the migration of the intact form of UFD2 was retarded in UVB-irradiated cells, indicating that UFD2 is also subject to other post-translational modifications upon transduction of certain signals (Figure 7A, and S. M. White, J. A. Mahoney, L. Casciola-Rosen and A. Rosen, unpublished results).

At sites of intracellular infection, granzyme B is released from perforin-containing granules of cytotoxic T lymphocytes or natural killer cells, and mediates the granule-induced cell death pathway. The effects of granule-induced cell death on UFD2 were investigated by co-culturing LAK cells with Fas-negative K562 human erythroblast cells [7]. LAK cell co-culture caused cleavage of UFD2, generating the 110 kDa fragment (Figure 7B). The same fragment was generated in the presence of the caspase 3/7 inhibitor DEVD-CHO, confirming that these caspases are not responsible for generation of this fragment. We verified that DEVD-CHO had indeed exerted its caspase inhibitory effect in this experiment as follows. When aliquots of the same samples were immunoblotted with an antibody to poly(ADP-ribose) polymerase (PARP), the 89 kDa caspase 3/7 fragment was detected in the LAK cell/K562 mix, but was not detected in reactions containing DEVD-CHO. These data confirm that UFD2 is efficiently cleaved in intact cells in response to a variety of different apoptotic signals delivered from either inside or outside the cell.

An intact N-terminal domain is necessary for functional UFD2 activity *in vitro*

To help determine the functional significance of granzyme B/caspase 6 cleavage of UFD2, recombinant UFD2 proteins, both full-length and the C-terminal domain lacking the first 123 amino acids (C-terminal), were produced using a baculovirus expression system (Figure 8A). These recombinant proteins were assayed for E3-like enzyme activity, i.e. polyubiquitination of themselves and/or exogenously added *E. coli* proteins (Figure 8B). As reported previously [23], full-length UFD2 catalysed robust attachment of ubiquitin both to itself and to numerous bacterial proteins. Omission of ubiquitin-activating (E1) and ubiquitin-conjugating (E2) proteins abolished nearly all staining, demonstrating the specificity of the assay system. In marked contrast, C-terminal UFD2 showed greatly attenuated polyubiquitination activity, failing to ubiquitinate both itself and exogenously added bacterial proteins (visible as a ladder of many labelled bands in the UFD2 full-length lane), thus demonstrating that functional enzymic activity has been lost in C-terminal UFD2. These data strongly suggest that the 123-amino-acid N-terminal domain removed by granzyme B/caspase 6 cleavage is required for UFD2 function.

DISCUSSION

We report here that a human homologue of yeast Ufd2p, a protein that in yeast catalyses the formation of elongated polyubiquitin chains on target proteins, is cleaved by caspases and granzyme B in apoptotic cells. Human UFD2 was identified using monospecific autoreactive serum from a patient with systemic autoimmune disease, and was specifically cleaved by both caspases and granzyme B in apoptotic cells and *in vitro*. Intriguingly, cleavage of UFD2 occurred exclusively in an N-terminal domain absent from yeast Ufd2p and other family members, but highly conserved among human UFD2 and two apparent vertebrate species orthologues. Enzymic activity was abolished in recombinant UFD2 corresponding to this cleavage product. These data suggest that the N-terminal domain of human UFD2 has an important role in UFD2 function, and that cleavage by aspartate-specific proteases might modulate that function.

The yeast Ufd proteins were described in 1995 as five structurally unrelated genes involved in ubiquitin fusion protein degradation [27]. While ubiquitin-tagged fusion proteins are usually rapidly degraded by yeast, mutations in UFD1–5 diminished markedly the efficiency of this pathway. Further analysis showed that Ufd2p enhanced ubiquitin chain elongation from only one of the two attachment sites, Lys^{48}, and had no effect on elongation from the other site, Lys^{29} [27]. A homologue of yeast *UFD2* called *nos*A was subsequently identified in *Dictyostelium discoideum*, which, when deleted, led to changes in the ubiquitination patterns of a subset of proteins [28]. Importantly, deletion of *nos*A led to developmental arrest at the tight aggregate stage, highlighting the fact that changes in ubiquitination of even a small subset of host proteins might have profound effects on fundamental functions, such as growth and development. The molecular role of yeast Ufd2p remained unknown until 1999, when Koegl and co-workers [18] showed that addition of purified Ufd2p (which they have termed E4) to *in vitro* ubiquitination reactions containing purified E1, E2 and E3 enzymes of the ubiquitin cascade rapidly and dramatically increased the length of ubiquitin chains attached to model substrates. Only these long ubiquitin chains are recognized by the proteasome for degradation. Yeast carrying a mutation in the *UFD2* gene showed increased susceptibility to some types of environmental stress, especially in combination with a mutation in the ubiquitin-binding protein RPN10 [18]. Interestingly, a recent sequence-alignment analysis of UFD2 family members indicated that the U box might represent a modified version of the RING finger domain found in a large subset of E3 ubiquitin ligases [29].

Site-directed mutagenesis identified two major cleavage sites for aspartate-specific proteases in UFD2 (Figure 5). Among the

caspases, caspase 6 cleaved the most efficiently, recognizing the tetrapeptide VDVD[123]. Granzyme B cleaved at this same site, also with high efficiency. Caspases 3 and 7 recognized the sequence MDID[109], cleaving approx. 10-fold less efficiently than caspase 6. Interestingly, methionine was not included in P_4 in the tetrapeptide scanning library that defined the specificity of group II caspases [15]. While the current studies demonstrate that this P_4 methionine residue can be recognized by caspases 3 and 7, this cleavage is more than two orders of magnitude less efficient than that for substrates with aspartate in P_4 (e.g. PARP; $k_{cat}/K_m = 5 \times 10^6\ M^{-1} \cdot s^{-1}$ [7]).

Strikingly, in a protein of 1173 amino acids, the caspase 3/7 and granzyme B cleavage sites are separated by only 14 amino acids. We and others have noted this same close spacing of caspase and granzyme B cleavage sites in a large number of proteins, including DNA-dependent protein kinase [7], topoisomerase I, Mi2 and Ki-67 [14], Bid [13] and DNA fragmentation factor-45 ('DFF45') [12]. This suggests that caspases and granzyme B may have evolved to cleave separate elements of a single, larger protein motif that functions in many essential cellular processes, including DNA replication, DNA repair, nucleosome remodelling, mRNA splicing, translation and apoptosis. Studies to define further such a motif are currently in progress.

Although both caspase 6 and granzyme B cleave UFD2 efficiently at the same site, mutagenesis of the two aspartate residues within the tetrapeptide recognition site (VDVD) showed that the fine substrate specificities of these two proteases are different (Figure 6), in that mutation of the P_3 aspartate to alanine (VAVD) has little effect on the efficiency of granzyme B cleavage, yet caspase 6 cleavage is almost completely abolished. The cleavage patterns of these mutants upholds the predictions of experiments performed with a combinatorial tetrapeptide scanning library [15]: namely, that alanine in the P_3 position is tolerated by granzyme B, but less well tolerated by caspase 6.

A wide variety of apoptotic stimuli led to human UFD2 cleavage in intact cells (Figure 7), including UV irradiation, protein kinase inhibition, Fas ligation and cytotoxic lymphocyte-mediated cell killing. UFD2 cleavage in the cytotoxic lymphocyte/K562 assay system is particularly striking: in the presence of sufficient DEVD-CHO to inhibit caspase 6, a large percentage of UFD2 is still cleaved, indicating that UFD2 is a direct substrate of granzyme B in intact cells. The wide variety of cell types and stimuli that exhibit this phenomenon suggests that UFD2 cleavage may play an important role in the apoptotic cascade.

Human UFD2 is a member of a family of proteins conserved from yeast through mammals. Although the role of yeast Ufd2p in long ubiquitin chain formation was elucidated recently [18], it is not yet clear whether this human UFD2 homologue serves the same function. Even in yeast, it is not known under what circumstances Ufd2p acts. Koegl and co-workers [18] suggest that only a subset of E3 ubiquitin ligases require E4/UFD2 for efficient polyubiquitination. The fact that UFD2-null yeast are viable [27] suggests that the proteasome pathway has redundant mechanisms to target proteins for proteasomal degradation. Human UFD2 differs from all lower eukaryote family members in that it possesses a unique 300 amino acid N-terminal extension. This region of the molecule has no similarity to other known sequences, except putative vertebrate orthologues of human UFD2 from mouse and zebrafish, which share $\geqslant 90\%$ sequence identity within the N-terminal region, including conservation of both aspartate-specific protease cleavage sites (Figure 2C). A paper published very recently [23] has shown that human UFD2 and other U-box-containing proteins have E3-like ubiquitin ligase activity, and established the requirement of the U box for enzymic activity. Our experiments demonstrate that disruption of the vertebrate-specific N-terminal domain, far removed from the putative catalytic U box, abrogates this E3-like activity.

In conclusion, we have cloned a human homologue of UFD2, which is cleaved by caspases and granzyme B, both *in vitro* and in cells treated with a variety of apoptotic stimuli. Human UFD2 shows similarity to yeast Ufd2p, a protein shown recently to catalyse formation of the long ubiquitin chains necessary for recognition and degradation of target proteins by the proteasome. The shared caspase 6/granzyme B cleavage site is immediately adjacent to a caspase 3/7 cleavage site within a novel N-terminal domain highly conserved in vertebrates. Cleavage of this putative UFD2 regulatory domain by caspases or granzyme B during apoptosis might influence the apoptotic cascade; for example, by abolishing E3 activity of UFD2 and thereby stabilizing pro-apoptotic factors. Defining the downstream targets of UFD2 is a major priority.

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REFERENCES

1 Jacobson, M. D., Weil, M. and Raff, M. C. (1997) Programmed cell death in animal development. Cell **88**, 347–354
2 Evan, G. and Littlewood, T. (1998) A matter of life and cell death. Science **281**, 1317–1322
3 Vaux, D. L. and Flavell, R. A. (2000) Apoptosis genes and autoimmunity. Curr. Opin. Immunol. **12**, 719–724
4 Nicholson, D. W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ. **6**, 1028–1042
5 Motyka, B., Korbutt, G., Pinkoski, M. J., Heibein, J. A., Caputo, A., Hobman, M., Barry, M., Shostak, I., Sawchuk, T., Holmes, C. F. et al. (2000) Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. Cell **103**, 491–500
6 Darmon, A. J., Nicholson, D. W. and Bleackley, R. C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. Nature (London) **377**, 446–448
7 Andrade, F., Roy, S., Nicholson, D., Thornberry, N., Rosen, A. and Casciola-Rosen, L. (1998) Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. Immunity **8**, 451–460
8 MacDonald, G., Shi, L., Vande, V. C., Lieberman, J. and Greenberg, A. H. (1999) Mitochondria-dependent and -independent regulation of granzyme B-induced apoptosis. J. Exp. Med. **189**, 131–144
9 Heibein, J. A., Goping, I. S., Barry, M., Pinkoski, M. J., Shore, G. C., Green, D. R. and Bleackley, R. C. (2000) Granzyme B-mediated cytochrome *c* release is regulated by the Bcl-2 family members bid and Bax. J. Exp. Med. **192**, 1391–1402
10 Sutton, V. R., Davis, J. E., Cancilla, M., Johnstone, R. W., Ruefli, A. A., Sedelies, K., Browne, K. A. and Trapani, J. A. (2000) Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. J. Exp. Med. **192**, 1403–1414
11 Alimonti, J. B., Shi, L., Baijal, P. K. and Greenberg, A. H. (2000) Granzyme B induces BID-mediated cytochrome *c* release and mitochondrial permeability transition. J. Biol. Chem. **276**, 6974–6982
12 Wolf, B. B., Schuler, M., Echeverri, F. and Green, D. R. (1999) Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. J. Biol. Chem. **274**, 30651–30656
13 Li, H., Zhu, H., Xu, C. J. and Yuan, J. (1998) Cleavage of Bid by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell **94**, 491–501
14 Casciola-Rosen, L., Andrade, F., Ulanet, D., Wong, W. B. and Rosen, A. (1999) Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. J. Exp. Med. **190**, 815–825
15 Thornberry, N. A., Ranon, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P. et al. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B: functional relationships established for key mediators of apoptosis. J. Biol. Chem. **272**, 17907–17911

16 Harris, J. L., Peterson, E. P., Hudig, D., Thornberry, N. A. and Craik, C. S. (1998) Definition and redesign of the extended substrate specificity of granzyme B. J. Biol. Chem. **273**, 27364–27373
17 Casciola-Rosen, L. A., Anhalt, G. and Rosen, A. (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J. Exp. Med. **179**, 1317–1330
18 Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U. and Jentsch, S. (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell **96**, 635–644
19 Sanghavi, D. M., Thelen, M., Thornberry, N. A., Casciola-Rosen, L. and Rosen, A. (1998) Caspase-mediated proteolysis during apoptosis: insights from apoptotic neutrophils. FEBS Lett. **422**, 179–184
20 Tschopp, J. (1994) Granzyme B. Methods Enzymol. **244**, 80–87
21 Stennicke, H. R. and Salvesen, G. S. (1999) Caspases: preparation and characterization. Methods **17**, 313–319
22 Greidinger, E. L., Miller, D. K., Yamin, T.-T., Casciola-Rosen, L. and Rosen, A. (1996) Sequential activation of three distinct ICE-like activities in Fas-ligated Jurkat cells. FEBS Lett. **390**, 299–303

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23 Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N. and Nakayama, K. I. (2001) U box proteins as a new family of ubiquitin-protein ligases. J. Biol. Chem. **276**, 33111–33120
24 Casciola-Rosen, L. A., Anhalt, G. J. and Rosen, A. (1995) DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. J. Exp. Med. **182**, 1625–1634
25 Stennicke, H. R., Jurgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D. et al. (1998) Pro-caspase-3 is a major physiologic target of caspase-8. J. Biol. Chem. **273**, 27084–27090
26 Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D. and Wong, W. W. (1997) Substrate specificities of caspase family proteases. J. Biol. Chem. **272**, 9677–9682
27 Johnson, E. S., Ma, P. C., Ota, I. M. and Varshavsky, A. (1995) A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. **270**, 17442–17456
28 Pukatzki, S., Tordilla, N., Franke, J. and Kessin, R. H. (1998) A novel component involved in ubiquitination is required for development of *Dictyostelium discoideum*. J. Biol. Chem. **273**, 24131–24138
29 Aravind, L. and Koonin, E. V. (2000) The U box is a modified RING finger – a common domain in ubiquitination. Curr. Biol. **10**, R132–R134