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Caspase-dependent Inactivation of Proteasome Function during Programmed Cell Death in *Drosophila* and Man*

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The caspase family of cysteine proteases plays a conserved role in the coordinate demolition of cellular structures during programmed cell death from nematodes to man. Because cells undergoing programmed cell death in nematodes, flies, and mammals all share common features, this suggests that caspases target a common set of cellular structures in each of these organisms. However, although many substrates for mammalian caspases have been identified, few substrates for these proteases have been identified in invertebrates. To search for similarities between the repertoires of proteins targeted for proteolysis by caspases in flies and mammals, we have performed proteomics-based screens in *Drosophila* and human cell lines undergoing apoptosis. Here we show that several subunits of the proteasome undergo caspase-dependent proteolysis in both organisms and that this results in diminished activity of this multicatalytic protease complex. These data suggest that caspase-dependent proteolysis decreases protein turnover by the proteasome and that this is a conserved event in programmed cell death from *Drosophila* to mammals.

The caspase family of cysteine proteases plays a conserved role in coordinating programmed cell deaths in multicellular organisms (1). In the nematode *Caenorhabditis elegans*, a single caspase, CED-3, is sufficient to promote all developmental related programmed cell deaths in this organism (2, 3). However, although the mechanism of activation of CED-3 by its adaptor CED-4 is relatively well understood, it remains unclear how CED-3 promotes cell death because few substrates for this caspase have been identified. A similar caspase-dependent route to apoptosis has also been elucidated in *Drosophila melanogaster* involving the CED-4-related protein DARK (also called dApaf-1/HAC-1) and the CED-3-related protease DRONC (4–8). Upon activation, DRONC promotes the activation of several additional fly caspases (9, 10), and these most likely act as the agents of cellular destruction in this organism. However, as in the nematode context, only a handful of fly caspase substrates, including the fly caspases themselves, the lamin homolog DmO, the caspase inhibitor DIAP-1, and the transcription factor Relish, have been reported to date (9–13). A CED-4-related caspase-adaptor protein, Apaf-1, has also been described in mammals, and this protein acts as a positive regulator of caspase-9 activation in response to diverse forms of cellular stress (14, 15). Upon activation within an oligomeric Apaf-1 complex (the apoptosome), caspase-9 propagates a proteolytic cascade involving six additional caspases (caspases-2, -3, -6, -7, -8, and -10), which cooperate to dismantle cellular structures and achieve the morphological and functional end points characteristic of apoptosis (16, 17).

Intensive study of the mammalian cell death-associated caspases has revealed that an array of cellular proteins are targeted for restricted proteolysis during apoptosis (18). To date, ~220 substrates for mammalian caspases have been identified, but it is likely that proteolysis of many of these substrates has minimal impact on cell viability, and they fail to be cleaved by caspases in other organisms. However, because the morphological and functional end points of apoptosis are remarkably similar from nematodes to man, this suggests that caspases probably cleave overlapping subsets of substrates in nematodes, flies, and mammals.

To address this question, we have used a proteomics-based approach to look for similarities between the repertoire of proteins targeted for caspase-dependent proteolysis during apoptosis of *Drosophila* S2 cells and the human Jurkat T cell line. This analysis revealed that multiple subunits of the multicatalytic proteasome complex undergo caspase-dependent proteolysis during apoptosis in both organisms. Moreover, proteasome function was observed to decline during CD95-induced apoptosis of Jurkat cells and actinomycin D-induced apoptosis of *Drosophila* S2 cells. These data suggest that caspase-mediated proteolysis decreases protein turnover by the proteasome and that this is a conserved event in programmed cell death from *Drosophila* to mammals.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Staurosporine, actinomycin D, and cycloheximide were purchased from Sigma. The caspase inhibitor Z[1]-VAD-fmk and fluorogenic caspase substrate DEVD-AMC were obtained from Bachem, UK. The AMC-coupled proteasome substrates (LLVY and LLE), ubiquitin-AMC, and the recombinant isopeptidase T (IsoT) hydrolase were sourced from Affiniti Research Products, Exeter, UK. All antibodies directed against proteasome α, β, 19 S, and 11 S regulator subunits were purchased from Affiniti. Anti-caspase-3, anti-gelsolin, and anti-Rho-GDI antibodies were purchased from BD Laboratories (UK). Anti-caspase-9 was purchased from Oncogene Research Products (UK). Anti-*Drosophila* cyclin B, Rho-1, and profilin were obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Ames. Anti-actin monoclonal antibody was from ICN.

Preparation of Cell-free Extracts—Cell-free extracts of Jurkat and

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[1] The abbreviations used are: Z, benzyloxycarbonyl; CEB, cell extract buffer; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Cyt *c*, cytochrome *c*; DTT, dithiothreitol; fmk, fluoromethyl ketone; IPG, immobilized pH gradient; IsoT, isopeptidase T; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PIPES, 1,4-piperazinediethanesulfonic acid; AMC, 7-amido-4-methylcoumarin.

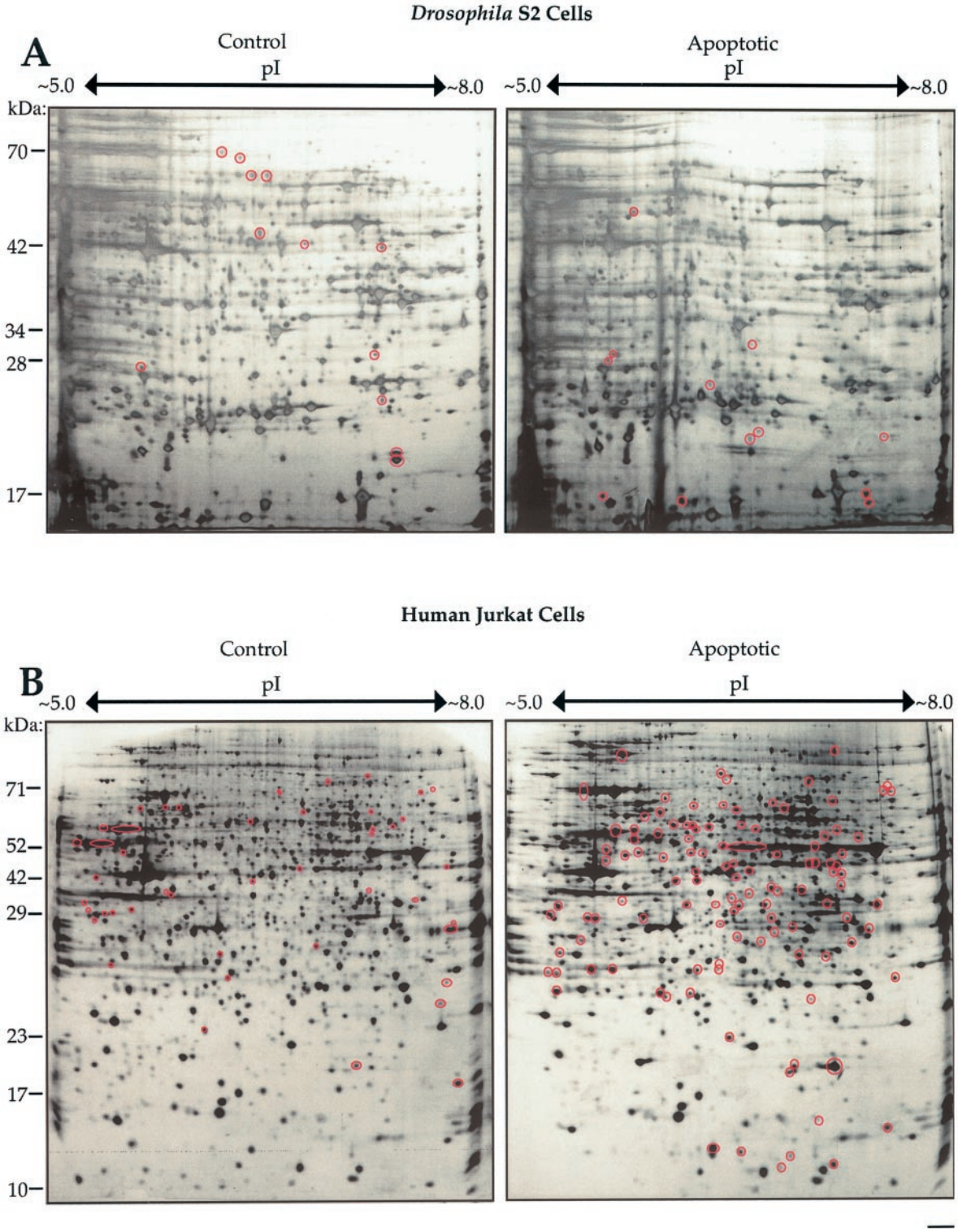

FIG. 1. **Detection of apoptosis-associated alterations to the proteome in *Drosophila* S2 and human Jurkat cells.** *A*, cytosolic extracts were generated from healthy *Drosophila* S2 cells (*left panel*) or cells exposed to 600 nM actinomycin D for 7 h (*right panel*). Protein samples (650 μg) were subjected to isoelectric focusing on pH 5–8 IPG strips followed by 12% second dimension SDS-PAGE. Proteins were then visualized by silver staining, as described under "Experimental Procedures." Protein spots that were altered reproducibly between control and apoptotic lysates are *circled*. A representative pH 5–8 gel is shown. The same analysis was also conducted using pH 3–6 IPG strips (data not shown). *B*, Jurkat cell-free extracts, either untreated (*left panel*) or treated with Cyt *c*/dATP to activate caspases (*right panel*), were incubated for 2 h at 37 °C. Protein samples were subjected to two-dimensional electrophoresis and visualized by silver staining, as described above. Protein spots that were altered reproducibly between control and apoptotic extracts are *circled*. A representative pH 5–8 gel is shown. The analysis was also conducted using pH 3–6 IPG strips (data not shown).

Drosophila S2 subline (D.Mel-2) cells were prepared as described previously (16, 17). Briefly, 5×10^8 cells were packed into a 2-ml Dounce homogenizer, and an equal volume of ice-cold cell extract buffer (CEB) was added (CEB: 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin). For generation of cell-free extracts from *Drosophila* S2 cells, 250 mM sucrose and 10 mM KCl were added to the CEB. Cells were allowed to swell in CEB for 20

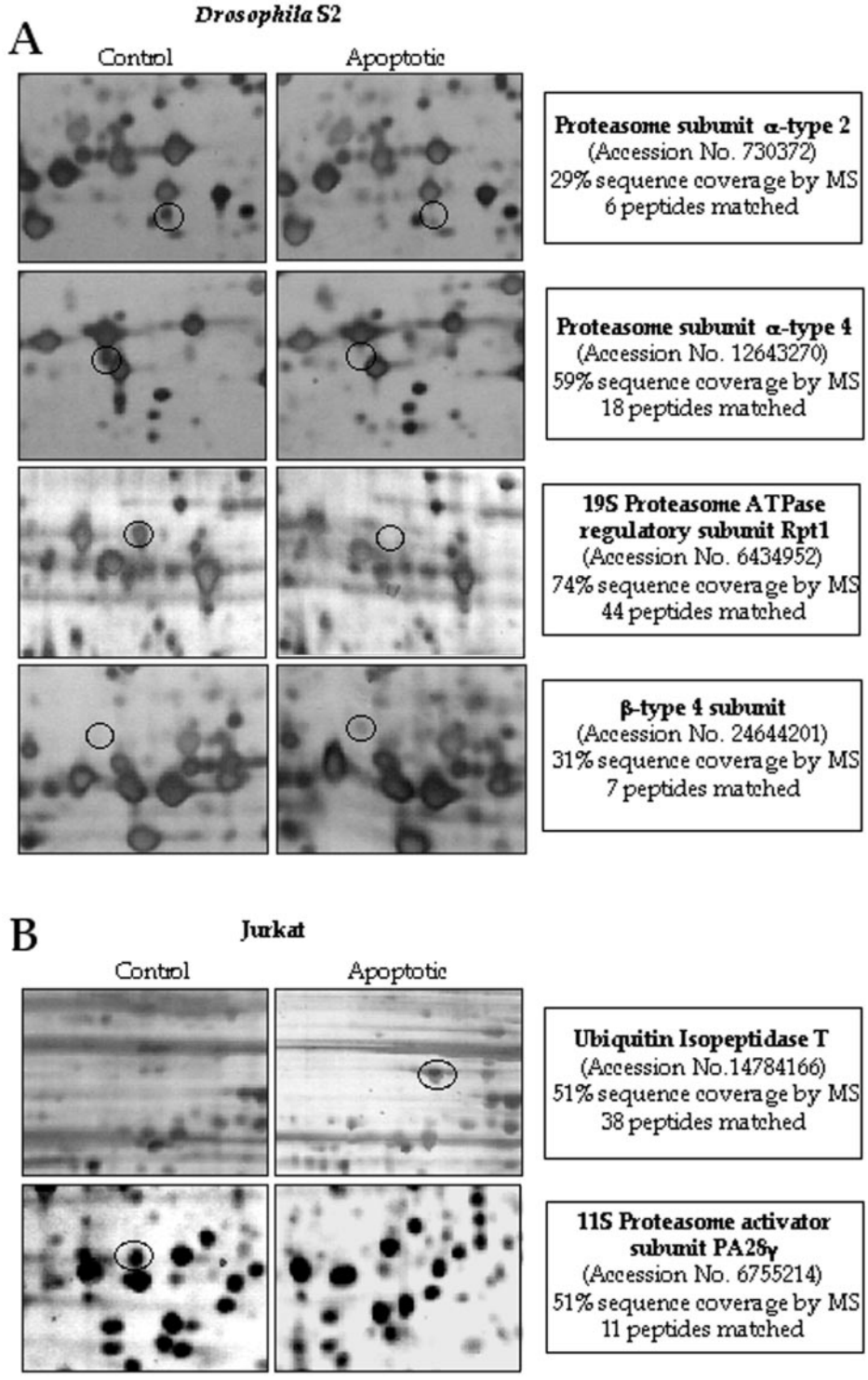

FIG. 2. **Cleavage of proteasome subunits and associated regulatory proteins in *Drosophila* S2 and human Jurkat cells.** *A*, enlarged areas of silver-stained two-dimensional gels comparing extracts from mock-treated (*left panel*) or apoptotic *Drosophila* S2 cells treated with 600 nM actinomycin D (*right panel*). Proteins that reproducibly disappeared during apoptosis or appeared as proteolytic fragments are indicated. *B*, enlarged areas of a silver-stained two-dimensional gels comparing control (*left panel*) or Cyt *c*/dATP-treated Jurkat cell-free extracts (*right panel*). Proteins that reproducibly disappeared during apoptosis or appeared as proteolytic fragments are indicated.

min on ice and were then lysed by homogenization with ~20–30 strokes of a B-type pestle. Crude extracts were then centrifuged for 15 min at 15,000 × *g* to remove nuclei, unbroken cells, and other debris.

Two-dimensional Gel Electrophoresis—For two-dimensional gel electrophoresis, 500 μg of cell-free extract in 350 μl of two-dimensional sample buffer (8 M urea, 4% CHAPS, 100 mM DTT, 0.05% SDS, 0.5% ampholyte 3–10, and a trace of bromphenol blue) was rehydrated into 17-cm immobilized pH gradient (IPG) strips (Bio-Rad). Passive sample rehydration into IPG strips was performed at room temperature overnight. Isoelectric point focusing was performed in a Bio-Rad Protean Isoelectric Point Focusing Cell under the following conditions: (i) linear voltage ramp to 500 V over 1 h; (ii) 5 h at 500 V; (iii) linear voltage ramp to 3,500 V over 5 h; and (iv) 12 h at 3,500 V. After isoelectric point focusing, the IPG strips were reduced and alkylated with 2% DTT and 2.5% iodoacetic acid, respectively, in 5-min incubations in an equilibration buffer that contained 6 M urea, 375 mM Tris-HCl, pH 8.8, 2% SDS, 20% glycerol. Strips were then mounted on 12% SDS-polyacrylamide gels using Easymelt agarose (Bio-Rad) and electrophoresed at 37.5 mA/gel in a Bio-Rad Protean II xi electrophoresis cell. Two-dimensional gels were stained using a mass spectrometry-compatible silver staining protocol that is based on a modification of the EMBL silver staining protocol (19).

In Gel Protein Digestion and Protein Identification by MALDI-TOF Mass Spectrometry—Protein spots were excised manually from two-dimensional gels. Gel pieces were incubated at room temperature on a shaking platform in oxidation buffer (15 mM $K_3Fe(CN)_6$, 50 mM $Na_2S_2O_3$) until the spots were completely destained. Gel pieces were then washed five times (5–10 min/wash) in 50% methanol and 10%

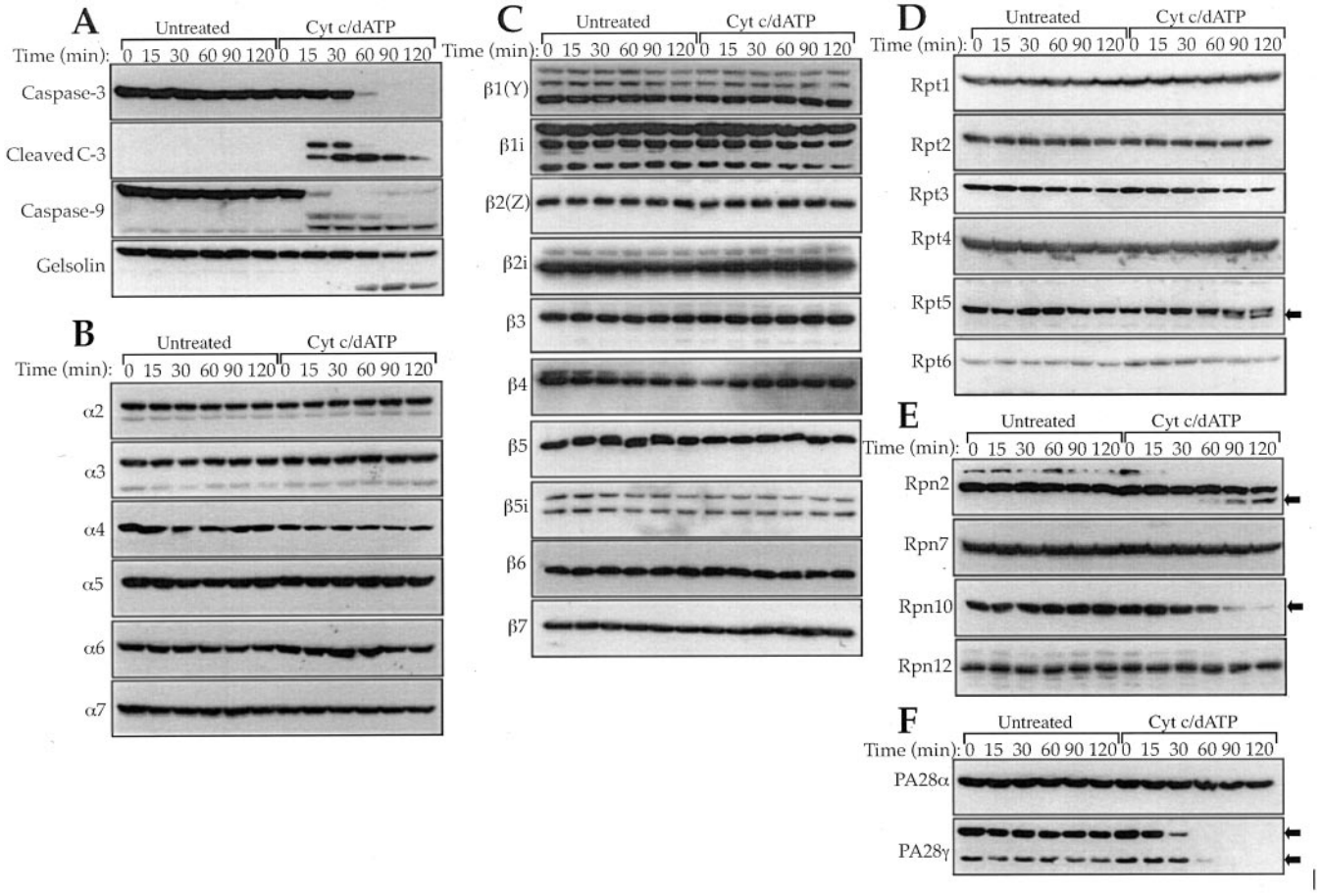

FIG. 3. **Caspase-dependent proteolysis of proteasome regulatory subunits.** Jurkat cell-free extracts were stimulated to undergo Cyt *c*/dATP-dependent caspase activation as described under "Experimental Procedures." Samples were removed at the indicated time points and analyzed by immunoblotting. *A*, Western blots were probed with antibodies specific to caspase-3, caspase-9, and gelsolin to confirm caspase activation. *B* and *C*, parallel samples were probed with a panel of antibodies specific to the indicated α or β core proteasome subunits. *D*, Western blots were probed with antibodies specific to the indicated 19 S ATPase proteasome regulatory subunits. The cleavage of subunit Rpt5 is indicated by an *arrow*. *E*, Western blots were probed with antibodies specific to the indicated 19 S non-ATPase proteasome regulatory subunits. The cleavage of Rpn2 and Rpn10 subunits is indicated by *arrows*. *F*, Western blots were probed with antibodies specific to the 11 S proteasome activator subunits PA28α and PA28γ. The cleavage of PA28γ is indicated by *arrows*.

acetic acid. Samples were then incubated in 50 mM NH_4HCO_3 for 5 min, prior to dehydrating in 100% acetonitrile. To dehydrate the pellets further, the acetonitrile was aspirated off, and the tubes were spun in a Speed-Vac (ThermoSavant) for 5 min at room temperature. For trypsin digestion, a 100 μg/ml aliquot of sequencing grade trypsin (Roche Applied Science) dissolved in 1 mM HCl was diluted 1:10 in digestion buffer (25 mM NH_4HCO_3, 0.1 *n*-octyl β-D-glucopyranoside). Typically, for low abundance silver-stained spots, 2 μl of trypsin solution (20 ng) was pipetted directly unto the desiccated gel piece. After allowing the gel piece to rehydrate for 5 min, a further 10 μl of digestion buffer was added, and samples were incubated overnight at 37 °C. After trypsin digestion, peptides were extracted twice into 40 μl of 66% acetonitrile and 0.1% trifluoroacetic acid in a sonicating water bath, followed by lyophilization in a Speed-Vac at room temperature. For mass spectrometric analysis, peptides were solubilized by sonication in 5 μl of 5% formic acid. Digested samples (0.5–1 μl) were applied to a Teflon-coated 96-well MALDI target plate (Applied Biosciences) followed by the addition of 0.5–1 μl of a 10 mg/ml matrix solution of α-cyano-4-hydroxycinnamic acid in 60% acetonitrile and 0.1% trifluoroacetic acid. Samples were allowed to air dry at room temperature before analysis in positive reflectron mode in a Voyager DE Pro MALDI mass spectrometer (Applied Biosciences).

Immunodepletion of Extracts—Jurkat cell-free extracts were immunodepleted of caspase-3 as described previously (16, 17). Briefly, 40 μl of a 50% slurry of protein A/G beads was coated with 5 μg of anti-caspase-3 antibody by rotation for 3 h at 4 °C. Antibody-coated beads were then washed in phosphate-buffered saline before adding to cell-free extracts. Extracts were immunodepleted overnight at 4 °C under constant rotation, followed by removal of antibody-coated beads by centrifugation.

Proteasome, Caspase, and IsoT Substrate Hydrolysis Assays—For synthetic peptide hydrolysis assays, ~2 μl of cytosolic extract in a final volume of 100 μl of CEB was added to wells of black 96-well Fluotrac 200 plates. AMC-coupled peptides (DEVD, LLE, LLVY) were then added to each well (to a final concentration of 50 μM), and liberation of free AMC was monitored for 1 h at 37 °C at excitation and emission wavelengths of 360 and 465 nm, respectively. AMC-coupled ubiquitin was used to measure the C-terminal hydrolase activity of full-length IsoT, caspase-3, and caspase-3-cleaved IsoT. IsoT (1 μM) was cleaved by 1 μM recombinant caspase-3 by coincubation in 20-μl reaction volumes for 2 h at 37 °C, using protease reaction buffer (20 mM PIPES-KOH, pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 10 mM DTT). Ubiquitin-AMC hydrolysis assays were carried out in IsoT buffer (50 mM HEPES, pH 7.8, 500 μM EDTA, 3 mM DTT) using a final concentration of 100 nM IsoT, 1.5 μM ubiquitin-AMC and, where appropriate, 5 μM z-VAD-fmk in a final reaction volume of 50 μl.

RESULTS

Proteomic Screens for Caspase Substrates in Drosophila and Human Cell Lines—To search for fly caspase substrates, we used *Drosophila* S2 cells, which are well established to undergo apoptosis in response to inhibitors of protein or RNA synthesis (7, 9, 11). S2 cells exposed to low concentrations of actinomycin D (600 nM) displayed characteristic features of apoptosis, such as extensive membrane blebbing and apoptotic body production within 7 h of exposure to this drug (data not shown). In addition, robust caspase activity, as assessed by hydrolysis of the synthetic caspase substrate DEVD-AMC, was readily detected

in apoptotic S2 cell lysates (data not shown). To identify *Drosophila* caspase substrates, lysates prepared from control and apoptotic S2 cells were normalized for protein loading and were analyzed by two-dimensional gel electrophoresis (Fig. 1*A*). Protein spots that were reproducibly altered in two-dimensional profiles from control *versus* apoptotic S2 cell lysates were then excised and identified by MALDI-TOF mass spectrometry. A similar analysis was also performed using cell-free extracts generated from human Jurkat T cells triggered to undergo apoptosome-dependent caspase activation by the addition of cytochrome *c* (Cyt *c*) and dATP to the extracts (14, 16). Control *versus* apoptotic Jurkat cell-free extracts were also analyzed by two-dimensional gel electrophoresis (Fig. 1*B*), and altered protein spots were identified by mass spectrometry.

A full analysis of the apoptosis-associated alterations that were observed in the *Drosophila* S2 and human Jurkat cell proteomes will be described elsewhere. However, of the ~2,000 most abundant *Drosophila* proteins examined using this approach, we identified 44 protein spots that either disappeared from apoptotic cells or that appeared as cleavage products in these cells (Fig. 1*A*). Similarly, ~160 protein spots were observed to undergo caspase-dependent modification in Jurkat cell-free extracts (Fig. 1*B*). Of the ~44 potential caspase substrates identified from *Drosophila* S2 cells, four of these were components of the proteasome complex and were identified as α type 2, α type 4, and β type 4 subunits of the core 20 S proteasome and subunit Rpt1 of the 19 S regulatory complex (Fig. 2*A*). In addition, we also identified the γ subunit of the 11 S proteasome activator complex (PA28γ) and the ubiquitin-specific protease IsoT as caspase substrates in Jurkat cell-free extracts (Fig. 2*B*). Collectively, these data suggested that proteasomes and ubiquitin-proteasome pathway enzymes are targeted by caspases during apoptosis of fly and mammalian cells.

Caspase-dependent Cleavage of Human Proteasome Subunits—To explore in more detail the possibility that the proteasome is subjected to extensive caspase-dependent proteolysis during apoptosis, we concentrated on the Jurkat system because antibodies are readily available against most of the human proteasome constituents, whereas few are available for their fly counterparts. It has been established previously that addition of Cyt *c* and dATP to Jurkat cell-free extracts triggers a cascade of caspase activation events involving all of the cell death-associated caspases (16). Using this system, we observed that several of the 19 S proteasome regulatory subunits (Rpt5/S6a, Rpn2/S1, and Rpn10/S5a) underwent cleavage upon triggering of caspase activation within Jurkat extracts (Fig. 3, *D* and *E*). Moreover, in agreement with our proteomic analysis of the same cells, the proteasomal activator PA28γ was also found to be cleaved in Jurkat extracts after Cyt *c*/dATP treatment (Fig. 3*F*). To confirm that apoptosis-associated proteolysis of the proteasome subunits was the result of direct caspase cleavage, Cyt *c*/dATP-treated extracts were incubated in the presence or absence of the broad specificity caspase inhibitor, Z-VAD-fmk. As shown in Fig. 4*A*, under conditions where caspase activation was blocked by Z-VAD-fmk, cleavage of all of the proteasome-associated subunits was completely abrogated.

Interestingly, although proteomic analyses indicated that three of the core 20 S particle subunits (α type 2, α type 4, and β type 4) were cleaved during apoptosis of *Drosophila* S2 cells (Fig. 2*A*), their human counterparts did not appear to be cleaved by caspases (Fig. 3, *B* and *C*). Thus, although the 26 S proteasome appears to be targeted for proteolysis by caspases in both organisms, different subunits appeared to be involved.

It is important to note that although none of the mammalian 20 S core particle subunits were cleaved by caspases, it remains possible that the 19 S regulatory subunits that underwent proteolysis in Jurkat cells may also be cleaved by *Drosophila* caspases during apoptosis. However, we were unable to explore this possibility because antibodies to these fly proteins were unavailable. Nonetheless, these data confirm that multiple subunits of the regulatory complex of the human proteasome are substrates for caspases activated during apoptosis.

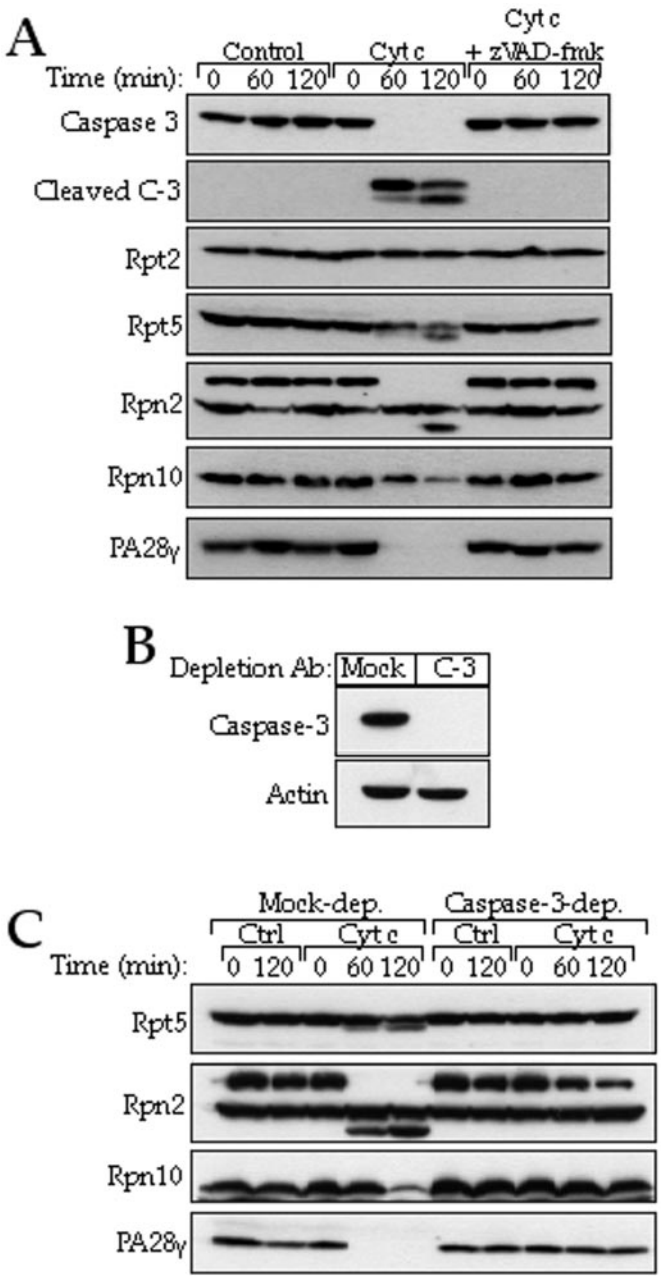

FIG. 4. **Cleavage of proteasome subunits requires caspase-3.** *A*, Jurkat cell-free extracts were treated with 50 μg/ml Cyt *c* and 1 mM dATP to initiate apoptosome-associated caspase activation, in the absence or presence of 5 μM Z-VAD-fmk. Samples were removed at the indicated time points and analyzed by immunoblotting with antibodies specific to caspase-3, Rpt2, Rpt5, Rpn2, Rpn10, and PA28γ. Note that the proteolysis of these subunits was blocked in the presence of Z-VAD-fmk. Subunit Rpt2 was included as a negative control. *B*, Jurkat cell-free extracts were immunodepleted using an anti-caspase-3 monoclonal antibody as described under "Experimental Procedures." Depletion of caspase-3 was confirmed by immunoblotting. An anti-actin blot serves as a loading control. *C*, mock-depleted and caspase-3-immunodepleted Jurkat cell-free extracts were treated with 50 μg/ml Cyt *c* and 1 mM dATP. Samples were removed at the indicated time points and analyzed by immunoblotting with antibodies specific to Rpt5, Rpn2, Rpn10, and PA28γ. Note that the proteolysis of all of these subunits was blocked in caspase-3-depleted extracts.

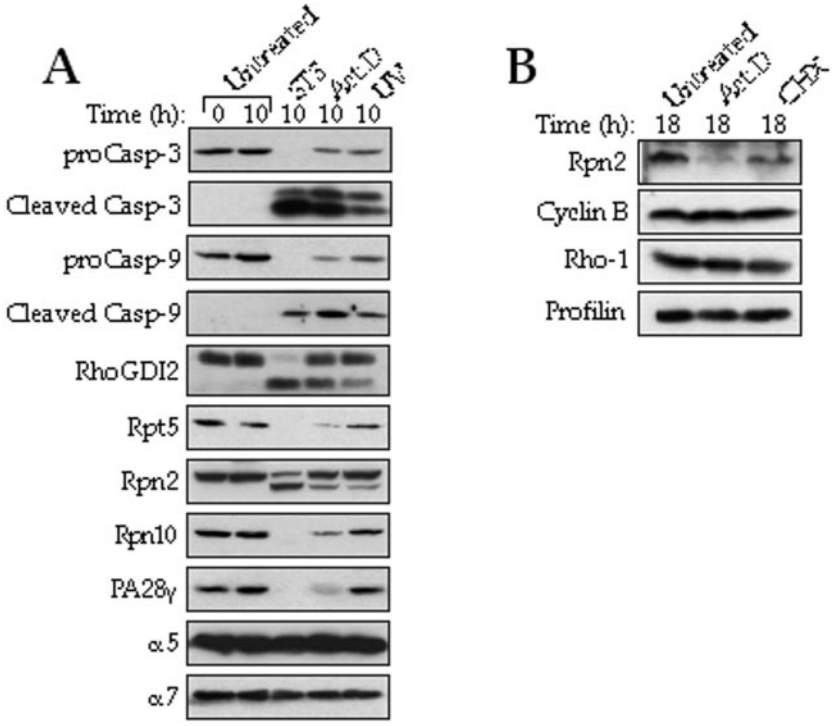

FIG. 5. **Apoptosis-associated proteolysis of 19 S proteasome subunits is stimulus-independent.** *A*, lysates were generated from Jurkat cells either mock-treated or exposed for 10 h to 500 nM staurosporine (*STS*), 30 μM actinomycin D (*Act.D*), or 4 min of UVB irradiation were immunoblotted with antibodies specific to caspases-3 and -9, RhoGDI2, and the proteasome subunits Rpt5, Rpn2, Rpn10, and PA28γ. Blots were probed with proteasome subunits α5 and α7 to serve as loading controls. *B*, *Drosophila* S2 cells were mock treated or exposed to 600 nM actinomycin D or 25 μM cycloheximide (*CHX*) for 18 h. Lysates were immunoblotted with antibodies specific to the human form of 19 S non-ATPase subunit Rpn2 as well as with antibodies specific to *Drosophila* cyclin-B, Rho-1, and profilin.

Proteolysis of Proteasome Regulatory Subunits Is Mediated by Caspase-3—Triggering of apoptosome activation by Cyt *c*/dATP is known to activate caspase-9 proximally, followed by caspases-3 and -7, which in turn activate caspases-6, -2, -8, and -10 (16, 20). To determine which of these caspases was responsible for proteolysis of the 19 S proteasome subunits, we generated Jurkat cell-free extracts that were immunodepleted of caspase-3 using a monoclonal antibody specific for this protease (Fig. 4*B*). Previous analyses have shown that caspase-3 plays a major role in the execution phase of apoptosis (17). Indeed, whereas mock-depleted cell-free extracts readily supported proteolysis of Rpt5, Rpn2, Rpn10, and PA28γ, immunodepletion of caspase-3 completely abrogated these events (Fig. 4*C*). Thus, caspase-3 appears to be responsible for the cleavage of several proteasome subunits during apoptosis.

Apoptosis-associated Proteolysis of 19 S Proteasome Subunits Is Stimulus-independent—To confirm that 19 S proteasome subunits were targeted for caspase-dependent proteolysis in response to diverse proapoptotic stimuli, Jurkat cells were triggered to undergo apoptosis by exposure to staurosporine, actinomycin D, or UV radiation (Fig. 5). In agreement with the observations made using Jurkat cell-free extracts, Rpt5 (S6a), Rpn2 (S1), Rpn10 (S5a), and PA28γ were cleaved during apoptosis in response to all of the above stimuli (Fig. 5*A*). Although no specific antibodies were commercially available against the *Drosophila* orthologs of these proteins, an antibody against human Rpn2 detected the loss of a band of the expected molecular mass in *Drosophila* S2 cells induced to undergo apoptosis by exposure to actinomycin D or cycloheximide (Fig. 5*B*). This suggests that, in addition to the subunits identified by proteomic analyses of *Drosophila* cells (Fig. 2*A*), Rpn2 (S1) may also be cleaved by caspases during apoptosis in the fly.

Proteasome Activity Is Impaired in Apoptotic Cells—To determine whether apoptosis-associated cleavage of proteasome subunits diminished the proteolytic activities of this complex, we assayed proteasome activity in control *versus* apoptotic Jurkat cells. Similar experiments were also performed on cell lysates derived from control *versus* apoptotic S2 cells. Cell extracts were generated in the presence of ATP to avoid dissociation of the 19 S regulatory components from the 20 S core particle. The ability of proteasomes in these extracts to hydrolyze the fluorogenic peptides LLE-AMC and LLVY-AMC (a measure of the peptidyl-glutamyl and chymotrypsin-like hydrolase activities of the proteasome, respectively) was then determined (Fig. 6). As shown in Fig. 6, *A* and *B*, extracts generated from apoptotic cells from both species exhibited robust caspase activity, as assessed by hydrolysis of DEVD-AMC, a synthetic caspase substrate. Significantly, extracts prepared from apoptotic Jurkat cells or from apoptotic *Drosophila* S2 cells both exhibited reduced LLEase and LLVYase activities compared with their viable counterparts (Fig. 6, *A* and *B*). These data suggest that caspase-dependent proteolysis of the proteasome during apoptosis results in a reduction of the proteolytic activities of this protein-degrading complex.

IsoT Is Inactivated through Caspase-3-mediated Proteolysis—Our proteomic analyses also identified the deubiquitinating enzyme IsoT as a potential caspase substrate (Fig. 2*B*). Although not a component of the proteasome complex, this enzyme is thought to be involved in the recycling of ubiquitin through hydrolysis of polyubiquitin chains to monomeric ubiquitin (21). Using purified recombinant IsoT, we confirmed that this protein is a substrate for caspase-3 and was cleaved to a fragment of ~90 kDa after incubation with this caspase *in vitro* (Fig. 6*C*). We then examined whether caspase-3-mediated proteolysis of IsoT altered the ability of this enzyme to hydrolyze ubiquitin-conjugated substrates. As shown in Fig. 6*D*, recombinant IsoT readily hydrolyzed ubiquitin-AMC, whereas caspase-3 was completely ineffective in the same assay. However, after exposure to caspase-3, IsoT exhibited dramatically reduced ubiquitin hydrolase activity (Fig. 6*D*). Thus, caspase-mediated proteolysis of IsoT is likely to attenuate the ability of the latter to dissemble polyubiquitin adducts during apoptosis, which is likely to have consequences similar to the inactivation of proteasome function, specifically, the accumulation of polyubiquitinated proteins.

DISCUSSION

Here we have provided evidence that several subunits of the 26 S proteasome complex are cleaved by caspases during apo-

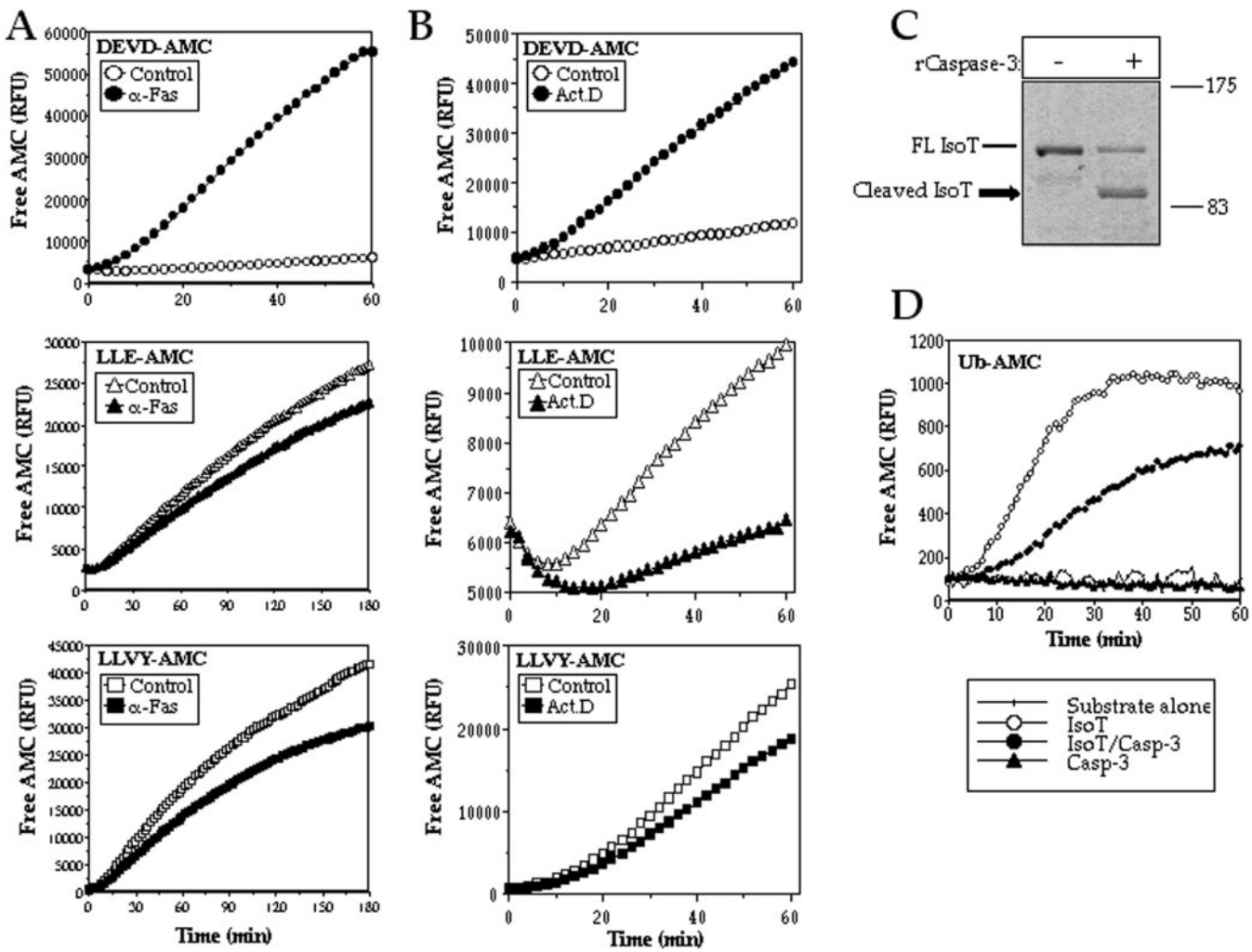

FIG. 6. **Diminished proteasome activity in apoptotic Jurkat and *Drosophila* cells.** *A*, Jurkat cells were either mock-treated or exposed to anti-Fas/CD95 monoclonal antibody (clone CH11, 100 ng/ml) for 5 h. Cell-free extracts, generated as described under "Experimental Procedures," were normalized for protein concentration and assayed for the ability to support hydrolysis of the fluorogenic caspase substrate peptide DEVD-AMC and the proteasome substrates LLE-AMC and LLVY-AMC. *B*, extracts from *Drosophila* S2 cells, either mock-treated or exposed to 600 nM actinomycin D (*Act.D*) for 7 h were assayed for the ability to cleave DEVD-AMC, LLE-AMC, and LLVY-AMC. *C*, purified recombinant human IsoT was incubated in the presence or absence or 1 μM recombinant caspase-3 for 2 h at 37 °C followed by analysis by SDS-PAGE and Coomassie Brilliant Blue staining. *D*, mock-incubated (*open circles*) or caspase-3-cleaved IsoT (*closed circles*) was tested for the ability to hydrolyze ubiquitin (*Ub*)-AMC. The final concentration of IsoT used in the assay was 100 nM. Recombinant caspase-3 (*triangles*) did not cleave ubiquitin-AMC, as shown.

ptosis in *Drosophila* and man. Although the proteasome subunits that are cleaved during apoptosis in the fly and in man appear distinct (with the possible exception of Rpn2), the functional consequences may be similar. In support of this idea, we also observed a decline in the peptidyl-glutamyl and chymotrypsin-like hydrolase activities of the proteasome during apoptosis of cells from both species. Collectively, these data suggest that proteasomes are conserved substrates of caspases from *Drosophila* to man and that the decline in proteasome function during apoptosis is at least partly attributable to caspase-mediated proteolysis of these complexes. Our data also provide a molecular basis for previous studies that have reported a decline in proteasome function and accumulation in polyubiquitinated proteins during apoptosis (22–24).

The functional consequences of proteasome inactivation during apoptosis is unclear. The proteasome complex is involved in multiple cellular functions such as coordination of cell division through the destruction of cyclins, nuclear factor-κB activation, protein turnover, antigen presentation, cell differentiation, and organelle biogenesis (25). In all of these situations, proteasomes are involved in the efficient degradation of proteins that are, in most cases, conjugated to polyubiquitin chains. The ubiquitin-proteasome pathway has also been implicated in the elimination of inhibitor of apoptosis proteins at the onset of apoptosis (8). Thus, targeting of proteasome complexes during apoptosis may serve to shut down diverse cellular functions that rely on the activity of this complex, thereby contributing to the demise of the cell.

RNA interference studies in *Drosophila* S2 cells have shown that perturbation of individual subunits of the 19 S regulatory complex, including those identified as substrates for caspases in the present study (Rpt5, Rpn2, and Rpn10), can result in spontaneous apoptosis (26). Furthermore, pharmacological inhibition of proteasome function is a well established and efficient means of triggering apoptosis (27–29). Inhibitors of proteasome function also sensitize transformed cells to the proapoptotic effects of conventional chemotherapeutics and radiation therapy and have significant clinical potential as a result (30). Collectively, these and other studies suggest that proteasomes are critical for normal cellular function and that interference with the activities of the proteasome has severe consequences for the cell. Thus, caspase-mediated proteolysis

of the proteasome may represent an important functional end point of apoptosis through disabling the main protein turnover machinery within the cell.

The present study represents the first comparative analysis of caspase substrates between *Drosophila* and man. Although it is often widely assumed that similar subsets of proteins undergo caspase-dependent proteolysis in nematodes, flies, and mammals, it is important to note that data to support this viewpoint are lacking at present. Although proteasome subunits represented only a small fraction of the proteins that underwent apoptosis-associated proteolysis in both organisms (see Fig. 1), our analyses to date have found surprisingly modest overlap between the proteins cleaved by caspases in the fly and mammals (data not shown). One explanation for this is that these analyses are not exhaustive and only examine the most abundantly expressed fraction of the fly and human proteomes that is accessible by two-dimensional gel analysis. In addition, our preliminary analysis has focused only on the repertoire of caspase substrates detected from a single cell type from each species. Therefore, further studies will be required to scrutinize in more detail the extent of conservation of caspase substrates between divergent species such as humans and flies. However, our work to date has identified a small number of proteins that are cleaved by caspases in both organisms, and these may represent particularly important caspase substrates (data not shown).

It is well established that a single *C. elegans* caspase, CED-3, is critical for all developmental related programmed cell deaths that take place in this organism (2, 3). However, it remains obscure how CED-3 coordinates cell death in the worm because physiological substrates for this caspase remain unknown. Further work is clearly required to identify conserved substrates of worm, fly, and mammalian caspases. Discovery of evolutionarily conserved caspase substrates is likely to shed considerable light upon the strategies used by caspases to kill cells.

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