THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 6, Issue of February 11, pp. 4663–4673, 2005
Printed in U.S.A.

Molecular Ordering of the Caspase Activation Cascade Initiated by the Cytotoxic T Lymphocyte/Natural Killer (CTL/NK) Protease Granzyme B*

Received for publication, September 22, 2004, and in revised form, November 23, 2004
Published, JBC Papers in Press, November 29, 2004, DOI 10.1074/jbc.M410915200

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Granzyme B is a major cytotoxic T lymphocyte/natural killer (CTL/NK) granule protease that can activate members of the caspase family of cysteine proteases through processing of caspase zymogens. However, the molecular order and relative importance of caspase activation events that occur in target cells during granzyme B-initiated apoptosis has not been established. Here, we have examined the hierarchy of granzyme B-initiated caspase activation events using a cell-free system where all caspases are present at physiological levels. We show that granzyme B initiates a two-tiered caspase activation cascade involving seven caspases, where caspase-3 is required for the second tier of caspase activation events. Using a two-dimensional gel-based proteomics approach we have also examined the scale of granzyme B-initiated alterations to the proteome in the presence or absence of effector caspase-3 or -7. These studies indicate that granzyme B targets a highly restricted range of substrates and orchestrates cellular demolition largely through activation of caspase-3.

Granzyme B (Gzm B)[1] is a serine protease found within the lytic granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (1–3). CTL/NK cells play important roles in tumor surveillance and anti-viral immunity and can promote killing of transformed or infected cells using several pathways (4, 5). One way in which CTLs and NK cells eliminate their targets is through degranulation of specialized lysosomal granules onto the surface of target cells (6). The latter event results in the entry of multiple CTL/NK granule proteins (granzymes) into the target cell, but how these granzymes coordinate death of the target is still poorly understood as substrates for several of the granzymes have yet to be found.

Two of the granzymes, Gzm A and Gzm B, have been intensively studied in recent years and a number of cellular substrates for these proteases have now been identified (7–11). It is well established that Gzm B can promote activation of members of the caspase family of cysteine proteases through proteolytic processing of several members of this family (12–19). However, because multiple caspases undergo proteolytic maturation in response to Gzm B, it has proved difficult to determine the primary target(s) of Gzm B in the caspase cascade and to unravel the hierarchy of caspase activation events that follow downstream. In contrast, Gzm A does not appear to target any of the caspases but rather targets proteins within the nuclear envelope and also activates a DNase complex through degradation of its inhibitor, SET (20, 21).

Gzm B can also promote caspase activation indirectly, through proteolysis of the Bcl-2 family protein, Bid (22, 23). Proteolysis of Bid by Gzm B results in the translocation of the C terminus of Bid to mitochondria where this protein provokes the release of mitochondrial cytochrome *c* into the cytosol (24, 25). Cytochrome *c* efflux from mitochondria results in the engagement of the apoptosome pathway to caspase activation and apoptosis (26). The caspase activation events in the apoptosome pathway have been intensively studied and are relatively well understood as a consequence (27–29).

The route chosen by Gzm B to initiate caspase activation (whether direct or via Bid) most likely depends on the effective concentration of this granzyme that is delivered by the CTL/NK into the target cell. At present, the concentration of Gzm B, or of the other granzymes, that is delivered into target cells during CTL/NK-mediated attack is unknown. However, studies using purified Gzm B suggest that nanomolar (50–100 nM) amounts of this granzyme are sufficient to engage the target cell death machinery (30). At these concentrations, Bid has been proposed as the preferred Gzm B substrate although this has been the subject of debate (23, 31, 32).

Irrespective of how Gzm B engages the caspase activation cascade, it also remains unclear what contributions individual caspases make to the terminal phase of apoptosis. Several caspases (caspase-3, -6, and -7) are thought to contribute to the destruction of the cell from within, probably through targeting hundreds of proteins for restricted proteolysis (33, 34). However, this aspect of apoptosis remains relatively poorly understood due to the sheer number of substrates that may be targeted by caspases.

Here, we have explored the hierarchy of caspase activation events that are initiated by Gzm B and have delineated a two-tiered caspase activation cascade where caspase-3 plays a required role in the activation of several other caspases. Furthermore, using proteomic analysis we also show that caspase-3 is the major target protease of granzyme B and is responsible for proteolysis of numerous cellular substrate proteins during the demolition phase of apoptosis.

* This work was supported by Science Foundation Ireland (PI1/B038). We have no conflicting financial interests. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Gzm B, granzyme B; CTL, cytotoxic T lymphocyte; NK, natural killer; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; AFC, amino-4-trifluoromethylcoumarin; IPG, immobilized pH gradient; CEB, cell extract buffer; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Cyt *c*, cytochrome c.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—For immunoblotting, antibodies specific to caspase-2, human caspase-3, murine caspase-3, caspase-6, caspase-7, and caspase-14 were purchased from BD Biosciences. Anti-caspase-8 antibodies were purchased from Apotech. Anti-caspase-9 antibodies were purchased from Oncogene Research Products. Anti-caspase-10 antibodies were purchased from Stratech. Anti-Bid antibodies were purchased from R+D Systems. For immunodepletion of Jurkat cell free extract, anti-caspase-3 and -7 antibodies were from BD Biosciences. Anti-caspase-6 antibodies were from Stratech. Anti-caspase-8 antibody clone 12F5 was from Apotech. Anti-caspase-9 antibody was purchased from Upstate Biotechnology. Purified human granzyme B was purchased from Calbiochem. Recombinant murine granzyme B was purchased from Sigma. Z-VAD-fmk and the fluorogenic caspase substrate Ac-DEVD-AFC were purchased from Bachem. Agarose-immobilized protein A/G was purchased from Santa Cruz Biotechnology. Immobilized pH gradient (IPG) strips were purchased from Bio-Rad. Unless stated otherwise, all other reagents were purchased from Sigma.

Preparation of Cell-free Extract and Induction of Granzyme B- or Apoptosome-dependent Caspase Activation—Cell-free extract was generated from exponentially growing healthy Jurkat T lymphocytic cells as described previously (27, 35). Briefly, ~5.0 × 108 Jurkat cells were harvested by centrifugation at 800 × *g* into a Dounce-type homogenizer. Two volumes of ice-cold cell extract buffer were 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 dithiothreitol, 250 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin), and the cells were allowed to swell for 15–20 min on ice. Cells were then lysed by homogenization with 10–15 strokes of a B-type pestle. Lysates were then clarified by centrifugation at 15,000 × *g* for 20 min to remove nuclei, mitochondria, and other cellular debris. Extracts were then frozen at −70 °C prior to use. For *in vitro* granzyme B-mediated activation of caspases, aliquots of cell-free extract were diluted 2-fold in CEB supplemented with a final concentration of 100 nM purified human granzyme B. Alternatively, to provoke apoptosome-dependent caspase activation, bovine heart cytochrome *c* and dATP were added to parallel reactions to final concentrations of 50 μg/ml and 1 mM, respectively.

Coupled in Vitro Transcription and Translation—^{35}S-Labeled proteins were generated using the TNT kit (Promega), as described previously (13, 27).

Immunodepletion of Caspases from Cell-free Extracts—Cell free extracts were immunodepleted of various caspases, essentially as described previously (35, 36). Briefly, 5 μg of antibody were precoupled to 30 μl of protein A/G-agarose beads in phosphate-buffered saline, pH 7.2 for 3–4 h at 4 °C. Bead-immobilized antibodies were then washed in CEB and incubated overnight with 50 μl of freshly made unfrozen cell free extract. The depleted extracts were then stored at −70 °C prior to use.

Fluorimetric Assessment of Caspase Activity—To measure the ability of Gzm B to stimulate executioner caspase activity, cell-free reactions were assembled in the presence or absence of 100 nM Gzm B. After incubating for 2 h at 37 °C, ~2.5 μl of the cell-free reactions were diluted to a final volume of 50 μl in CEB. An equal volume of DEVD-AFC was added to a final concentration of 50 μM, and the liberation of free AFC was monitored for 1 h at 37 °C in a fluorimeter using excitation and emission wavelengths of 430 and 535 nm, respectively.

Two-dimensional Gel Electrophoresis—For proteomic analysis, 350 μg of cell free extract solubilized in 350 μl 2D sample buffer (8 M urea, 4% CHAPS, 100 mM dithiothreitol, 0.05% SDS, 0.5% ampholyte 3–10 and a trace of bromphenol blue) were rehydrated passively into 17-cm pH 5–8 IPG strips. Isoelectric focusing was performed in a Bio-Rad protean isoelectric focusing cell under the following conditions: 1) linear voltage ramp to 500 V over 1 h, 2) 5 h at 500 V, 3) linear voltage ramp to 3500 V over 5 h, and 4) 12 h at 3500 V. Following isoelectric focusing, the IPG strips were reduced for 5 mins each in equilibration buffer (6 M urea, 375 mM Tris-HCl, pH 8.8, 2% SDS, 20% glycerol) containing 2% dithiothreitol and then alkylated in equilibration buffer instead containing 2.5% indole-3-acetic acid. Strips were then mounted on 12% SDS-PAGE gels and electrophoresed at 37.5 mA per gel in a Bio-Rad Protean II xi electrophoresis cell (Bio-Rad). Two-dimensional gels were stained using a mass spectrometry-compatible silver staining protocol (37).

Protein Identification by MALDI-TOF Mass Spectrometry—Protein spots excised from two-dimensional gels were incubated 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 extensively in 50% methanol, 10% acetic acid and equilibrated in 50 mM NH_4HCO_3 followed by dehydration in 100% acetonitrile. After drying in a Speed-Vac (Thermo Savant), 25–50 ng of trypsin in digestion buffer (25 mM NH_4HCO_3, 0.1 *n*-octyl β-D-glucopyranoside) were allowed to rehydrate directly into the gel piece for 5 min. A further 10 μl of digestion buffer were then added and samples were incubated overnight at 37 °C. The following day, peptides were extracted twice into 40 μl 66% acetonitrile, 0.1% trifluoroacetic acid in a sonicating water bath and lyophilized in a Speed-Vac at room temperature. For mass spectrometric analysis, peptides were solubilized in 5 μl of 5% formic acid. Samples (0.5–1 μl) were applied to a Teflon-coated 96-well MALDI target plate (Applied Biosciences, UK), followed by the addition of 0.5 to 1 μl of a 10 mg/ml matrix solution of α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, 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). After applying a filter to remove common background contaminant peaks, the de-isotoped spectra were submitted to the MS-Fit and MASCOT web-based data bases for protein identification.

RESULTS

Gzm B Promotes Activation of Multiple Caspases—To explore the Gzm B-initiated caspase activation cascade, we used a well established cell-free system based upon cytosolic extracts of Jurkat T lymphocytic cells (13, 27, 38, 39). As shown in Fig. 1*A*, addition of nanomolar amounts (100 nM) of purified human Gzm B to Jurkat cell-free extracts triggered the processing of multiple caspases to their signature active forms, with the exception of caspase-14. For comparison, cell-free extracts were also triggered to undergo caspase activation via the well characterized apoptosome pathway by addition of cytochrome *c* (Cyt c)/dATP to the extracts (Fig. 1*A*). These experiments revealed that caspase-2, -3, -6, and -7 were activated with similar kinetics in the granzyme B- and Cyt c/dATP-initiated pathways (Fig. 1). However, the kinetics of caspase-8 and caspase-10 processing were significantly faster in response to Gzm B, suggesting that these caspases were activated earlier in the Gzm B-driven cascade (Fig. 1*A*). As reported previously, Cyt c/dATP triggered processing of caspase-9 predominantly to the p35 form of the enzyme, indicating that the majority of active caspase-9 was derived from autoprocessing (Fig. 1*A*; Refs. 27 and 40). In sharp contrast, the p37 form of caspase-9 was the predominant species observed in the presence of Gzm B. Because this site has previously been identified as a caspase-3 processing site on caspase-9 (27, 40), this suggested that caspase-3 was responsible for the majority of caspase-9 processing seen in the Gzm B pathway (Fig. 1). Thus, although Gzm B and Cyt c/dATP activated the same repertoire of caspases, the kinetics and hierarchy of proteolytic events clearly differed between these distinct caspase activation mechanisms.

Direct Processing of Caspase-3, -7, -8, and -10 by Gzm B—Because certain caspases can activate other members of the caspase family, it was clearly possible that some of the caspase activation events triggered by Gzm B were indirect. To explore this possibility, cell-free reactions were carried out in the presence or absence of the polycaspase inhibitor, Z-VAD-fmk, to separate Gzm B-dependent and caspase-dependent processing events (Fig. 1*B*). As reported previously, addition of Z-VAD-fmk arrested the autoproteolytic maturation of caspase-3, as indicated by a p24 fragment corresponding to the caspase-3 large subunit/prodomain (Fig. 1*B*; Ref. 13). More importantly, this analysis revealed that the processing of caspase-2, -6, and -9 was completely arrested in the presence of Z-VAD-fmk (Fig. 1*B*). Thus, contrary to previous reports (14, 17, 18), Gzm B appears unable to directly process the latter caspases under conditions where processing of several other caspases is unaffected. These data suggest that only caspase-3, -7, -8, and-10 are direct targets of Gzm B.

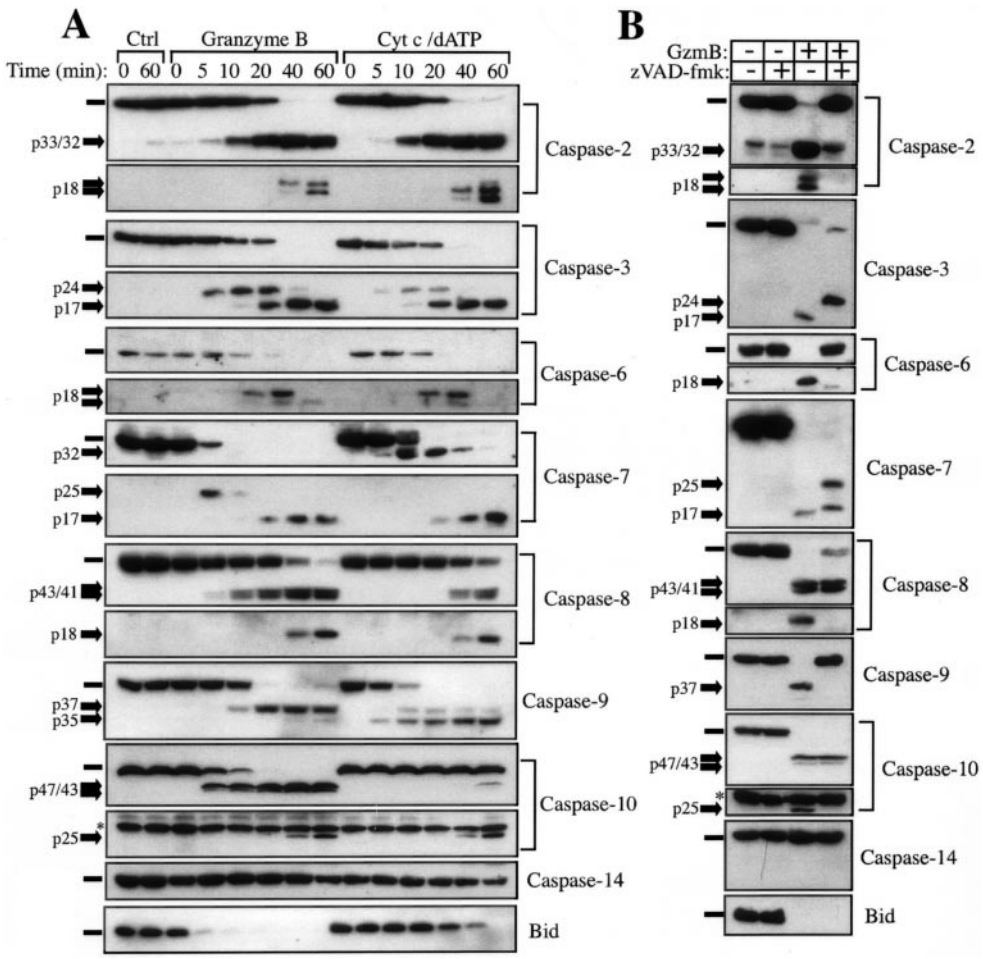

FIG. 1. **Granzyme B directly activates a subset of caspases and indirectly activates several others.** *A*, time course analysis of Gzm B *versus* cytochrome *c*-initiated caspase activation in Jurkat cell-free extracts. Cell-free extracts, either untreated or supplemented with 100 nM human Gzm B, or 50 μg/ml cytochrome *c*, 1 mM dATP, were incubated 37 °C for the indicated times. Protein samples (approximately 75 μg/lane) were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies. *Ctrl*, control. *B*, analysis of Gzm B-mediated caspase processing in the presence of a broad-spectrum caspase inhibitor. Jurkat cell-free extracts, supplemented with human Gzm B as indicated, were assembled in the absence or presence of the polycaspase inhibitor, Z-VAD-fmk (5 μM final concentration). Reactions were incubated for 1 h at 37 °C and then subjected to SDS-PAGE. The processing status of various caspases was examined by immunoblotting with the indicated antibodies. *Bars* indicate full-length caspase proforms, *arrows* indicate processed forms of caspases and their processing intermediates, and *asterisks* indicate nonspecific bands.

Relative Susceptibility of Caspases Versus Bid to Gzm B Proteolysis—Although Gzm B can directly process several caspases, much evidence also indicates that the BH-3-only molecule Bid is an important substrate for Gzm B (22, 23, 31). Indeed, Bid may be a significantly better substrate for Gzm B than either caspase-3 or caspase-8 (23). However, a recent study has proposed that Bid proteolysis during Gzm B/perforin-mediated killing may be an indirect event that occurs as a consequence of solubilization of endosomes by detergents during cell lysis (32). To date, the relative sensitivity of Bid *versus* the caspases as substrates for GzmB has not been examined comprehensively or in a setting where all of these proteins are present at physiological levels.

To explore this question, we titrated human Gzm B into Jurkat cell-free extracts in the presence of Z-VAD-fmk to preclude the possibility that caspase-dependent amplification events would complicate the analysis (Fig. 2*A*). Of the caspases examined, caspase-7 was found to be the most susceptible to Gzm B-mediated proteolysis and was still partially processed in the presence of 12 nM Gzm B (Fig. 2*A*). Bid was also highly susceptible to Gzm B-mediated proteolysis and, similar to caspase-7, was also cleaved at concentrations of 12 nM Gzm B (Fig. 2*A*). In contrast, caspase-3, -8, and -10 were markedly less sensitive to Gzm B-mediated proteolysis, with concentrations of 50–100 nM Gzm B being required to provoke significant maturation of these proteases (Fig. 2*A*). Thus, under conditions where Gzm B substrates are present at physiological levels, Bid and caspase-7 exhibited broadly similar sensitivities to Gzm B-mediated proteolysis. However, because caspase-7 cannot amplify the caspase activation cascade by processing other caspases (27), Bid is probably the most relevant target of Gzm B when the latter is delivered into target cells at limiting concentrations.

We also explored whether human and murine Gzm B exhibited a similar preference for proteolysis of Bid relative to caspase-3. Thus, we added murine Gzm B into cell-free extracts derived from murine J774 macrophages and compared the efficiency of proteolysis of Bid and caspase-3 within these extracts (Fig. 2*B*). In sharp contrast to what we observed using human Gzm B and cell-free extracts derived from human Jurkat cells, murine Gzm B preferentially cleaved caspase-3 rather than Bid in J774 cell-free extracts (Fig. 2*B*). This observation suggests that mouse and human Gzm B differ significantly in their substrate preferences. Furthermore, this observation was confirmed in experiments using murine Gzm B within the context of human cell free extract (Fig. 2*C*). As shown in Fig. 2*C*, although murine Gzm B displayed reduced activity toward human substrates as compared with murine

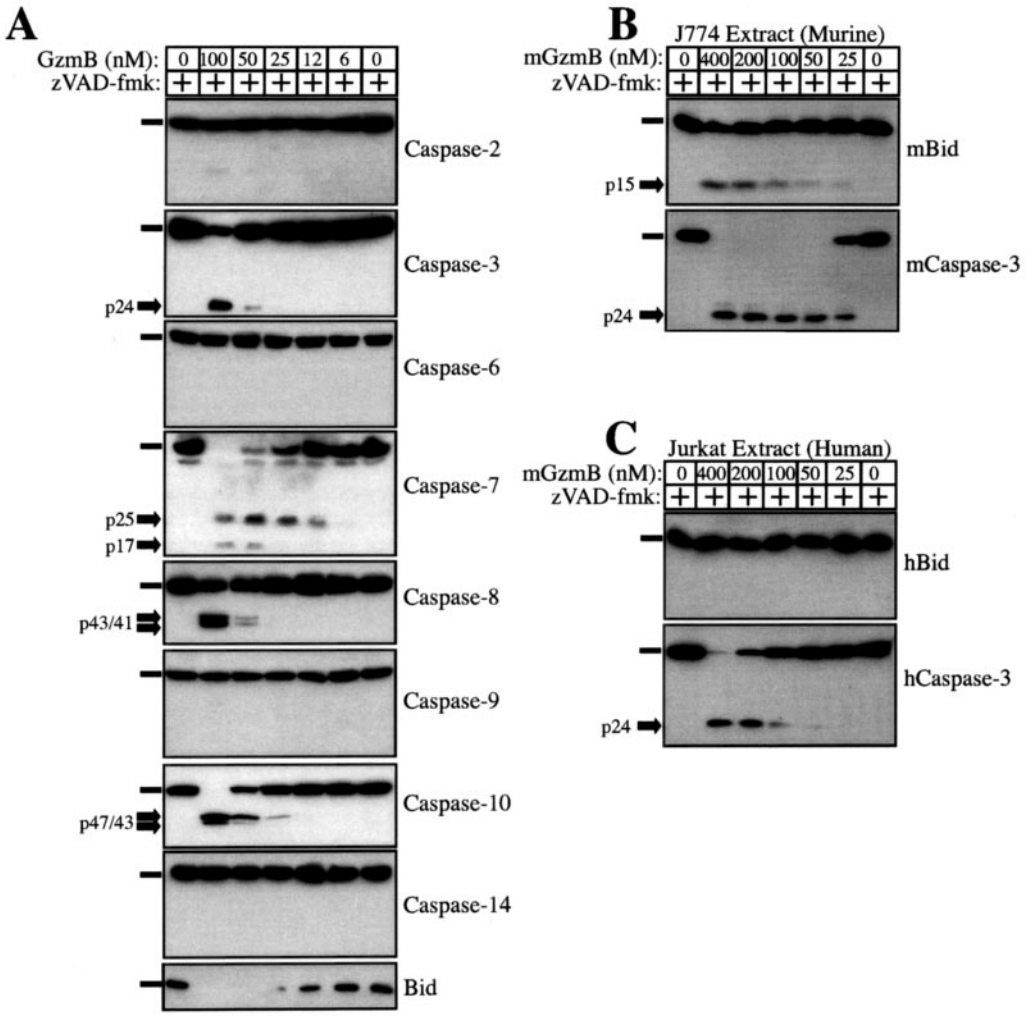

FIG. 2. **Bid is preferentially cleaved by human, but not murine, granzyme B.** *A*, Jurkat cell-free extracts containing 5 μM Z-VAD-fmk were exposed to the indicated concentrations of purified human Gzm B for 1 h at 37 °C. Samples were then subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies. *B*, J774 cell-free extracts containing 5 μM Z-VAD-fmk were exposed to the indicated concentrations of murine Gzm B for 1 h at 37 °C. Samples were subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies. *C*, Jurkat cell-free extracts containing 5 μM Z-VAD-fmk were exposed to the indicated concentrations of murine Gzm B for 1 h at 37 °C. Samples were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies.

substrates (Fig. 2*B*), the enzyme still exhibited a distinct preference for caspase-3 as opposed to Bid (Fig. 2*C*). By sharp contrast, human Gzm B cleaved Bid much more efficiently than caspase-3 (Fig. 2*A*). Taken together, these data suggest that the inability of murine GzmB to cleave Bid derives from differences in the enzymatic specificities between the murine and human enzymes, given that the behavior of murine Gzm B is similar in both human and murine extracts. Interestingly, two of the amino acids that represent determinants of the S2′ pocket subsite are not conserved between human and mouse or rat Gzm B, suggesting a possible basis for the observed differences in the specificities of the enzymes (41).

Caspase-3 Disseminates the Gzm B-triggered Caspase Activation Cascade—Because Gzm B was not directly responsible for the processing of procaspase-2, -6, and -9 (Fig. 1*B*), we next sought to identify the caspase(s) responsible for these downstream amplification events. To address this question, we used an immunodepletion approach to generate a panel of cell free extracts devoid of specific caspases, as described under "Experimental Procedures" (27, 35, 36). As shown in Fig. 3*A*, this approach specifically depleted individual caspases from extracts. We then assessed the ability of these depleted extracts to support Gzm B-initiated caspase activation events (Fig. 3*B*). As anticipated from the previous experiments, proteolytic processing of caspase-3, -7, -8, and -10 was not affected by immunodepletion of other caspases, confirming that these caspases are direct substrates for Gzm B (Fig. 3*B*). However, in cell-free extracts devoid of caspase-3, Gzm B-initiated processing of caspase-2, -6, and -9 was completely abrogated, indicating that caspase-3 was required for processing of these enzymes (Fig. 3*B*). In contrast, depletion of caspase-6, -7, or -9 did not affect Gzm B-initiated processing of any other caspase (Fig. 3*B*). Thus, upon activation by Gzm B, caspase-3 disseminates the caspase cascade by proteolytically processing caspase-2, -6, and -9.

Full Maturation of Caspase-8 and -10 Requires Caspase-3—As shown in Fig. 1*A*, Gzm B initially promoted the processing of caspase-8 to a p43/p41 doublet and at later timepoints the caspase-8 large subunit (p18) was detected. This processing pattern is consistent with an initial step that liberates the small subunit from the caspase-8 proenzyme, followed by a second proteolytic step that separates the caspase-8 prodomain from the p18 large subunit (Fig. 1). Because addition of Z-VAD-fmk to Gzm B-treated cell-free reactions blocked the appearance of the p18 subunit of caspase-8 (Fig. 1*B*), this suggested that removal of the prodomain from the p18 subunit was caspase-dependent. Similarly, the removal of the caspase-10 prodomain from the large subunit of this caspase was also blocked in the

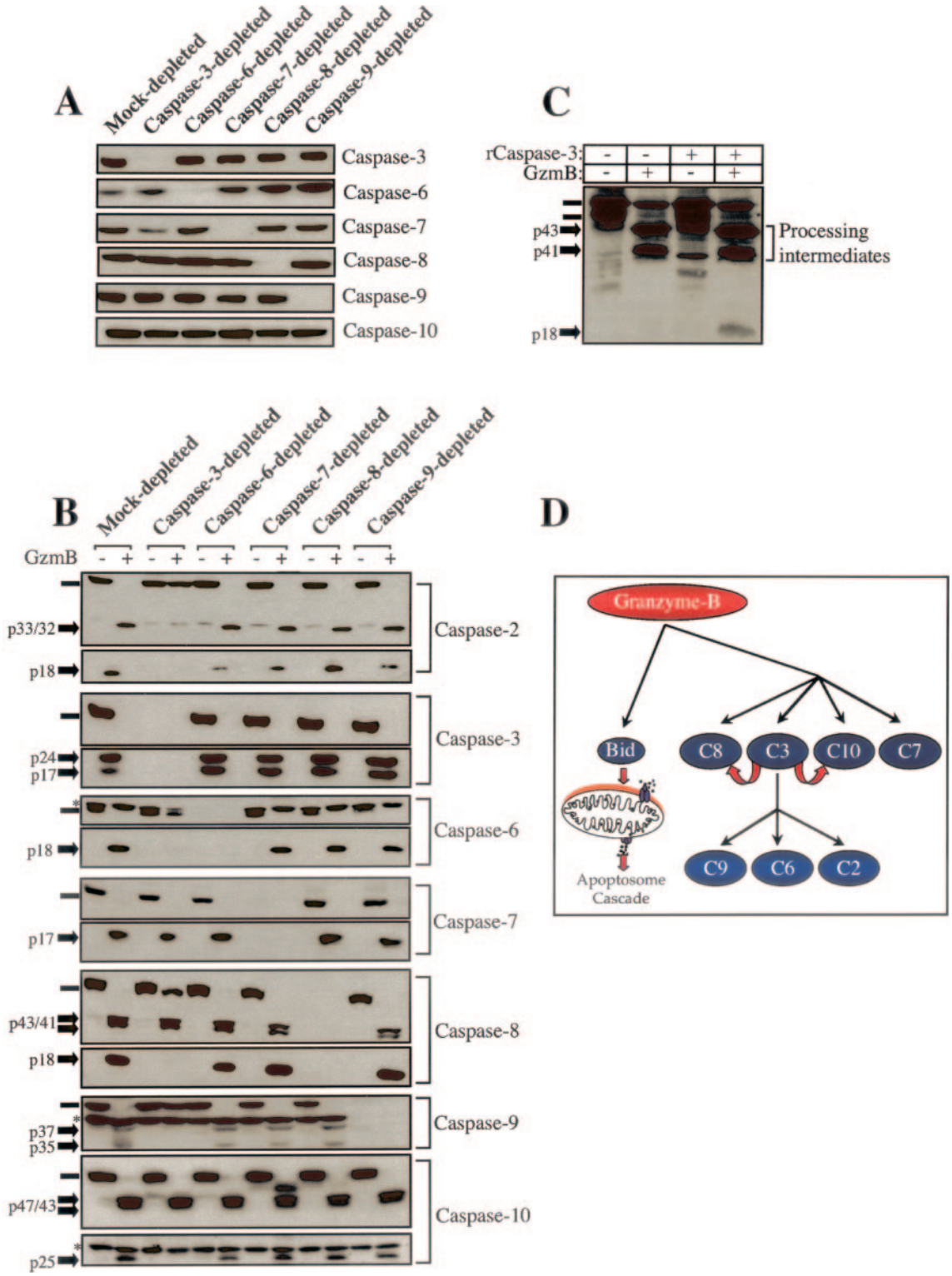

FIG. 3. **Analysis of caspase processing events in extracts immunodepleted of specific caspases reveals a role for caspase-3 in disseminating the caspase cascade.** *A*, Jurkat cell-free extracts were depleted using monoclonal antibodies specific to the individual caspases, as detailed under "Experimental Procedures." Depletion of specific caspases was confirmed by SDS-PAGE, followed by immunoblotting with the indicated antibodies. *B*, the cell extracts from *A* were supplemented with 100 nM human Gzm B, as indicated and incubated for 2 h at 37 °C. Samples were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies. *C*, *in vitro* translated caspase-8 was incubated in CEB in the presence or absence of purified human Gzm B (100 nM) or recombinant caspase-3 (1 μM) for 2 h at 37 °C. Samples were visualized by SDS-PAGE, followed by fluorography. *D*, schematic model of the Gzm B-triggered proteolytic cascade. Gzm B directly processes caspase-3, -7, -8, and -10. In this pathway, caspase-2, -6, and -9 are processed by caspase-3. Note that the second step of caspase-8 and caspase-10 maturation is mediated by caspase-3. Gzm B can also promote apoptosome-dependent activation of caspases via proteolysis of Bid, which promotes mitochondrial cytochrome *c* release and assembly of the Apaf-1/caspase-9 apoptosome.

presence of Z-VAD-fmk (Fig. 1*B*). Thus, full maturation of caspase-8 and -10, although initiated by Gzm B, are caspase-dependent.

The panel of cell-free extracts immunodepleted of individual caspases also enabled us to to identify the caspase responsible for removal of the prodomains of caspase-8 and -10. As shown

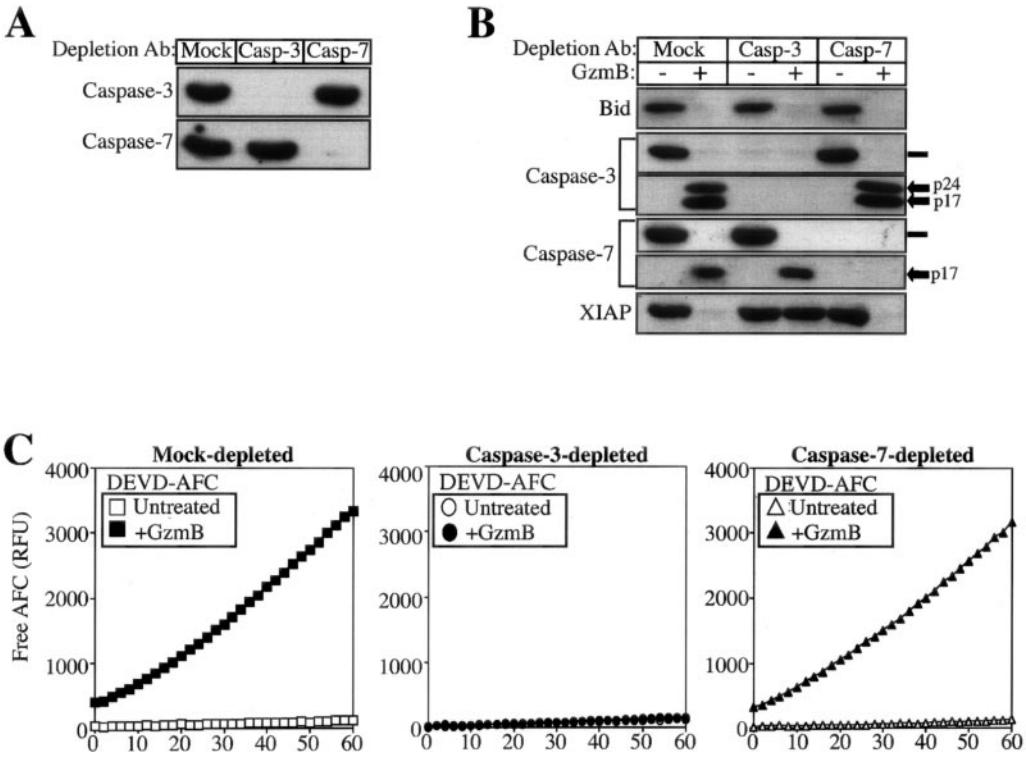

FIG. 4. **Essential requirement for caspase-3 in Gzm B-induced DEVDase activity.** *A*, Jurkat cell-free extracts were immunodepleted using monoclonal antibodies specific to caspase-3 and -7. Depletion of the caspases was confirmed by immunoblotting with the indicated antibodies. *B*, immunodepleted extracts from *A* were incubated for 2 h at 37 °C in the absence or presence of 100 nM human Gzm B, as indicated. Samples were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies. *C*, samples of the extracts from *B* were assessed for their ability to hydrolyze the fluorogenic caspase substrate DEVD-AFC as described under "Experimental Procedures."

in Fig. 3*B*, in mock-depleted extracts, or in extracts depleted of caspase-6, -7, or -9, Gzm B-initiated processing of caspase-8 and -10 remained unaffected. However, in extracts depleted of caspase-3, the processing of both caspase-8 and -10 was arrested at the ~45 kDa intermediate, similar to the effects seen with Z-VAD-fmk (Fig. 3*B*). This suggests that after initial processing by Gzm B, caspase-3 removes the N-terminal prodomain from caspase-8 and -10.

We also confirmed that caspase-3 was required for the complete maturation of caspase-8 using a different system. Here, we incubated *in vitro* transcribed and translated caspase-8 in the presence of Gzm B, either alone or in combination with recombinant caspase-3 (Fig. 3*C*). These experiments confirmed that the p18 subunit of caspase-8 was only produced when both Gzm B and caspase-3 were present (Fig. 3*C*). Thus, caspase-3 is required for full maturation of caspase-8 and -10 in the Gzm B-initiated cascade.

The Gzm B-initiated Caspase Activation Cascade—Taken together these data indicate that Gzm B initiates a two-tiered series of caspase activation events within target cells (Fig. 3*D*). Upon entry into the target, Gzm B is likely to directly process caspase-3, -7, -8, and -10 in parallel. Caspase-3 then undergoes a second maturation step involving the autocatalytic removal of its own prodomain (13). Fully active caspase-3 can then disseminate the caspase cascade by processing caspase-2, -6, and -9 and assists Gzm B in the maturation of caspase-8 and -10 (Fig. 3*D*).

Caspase-3 Plays a Central Role as an Effector Caspase within the Gzm B-initiated Caspase Cascade—Upon activation, caspases are thought to orchestrate the apoptotic phenotype through proteolysis of numerous caspase substrates (33, 34, 42). However, caspases are highly specific proteases and vary significantly in their substrate preferences and in the number of proteins they are likely to target during apoptosis (36, 43). The upstream or initiator caspases (such as caspase-8 and -9) appear to have a very restricted range of substrates and largely play a role in amplifying and disseminating caspase activation cascades. Caspase-3, -6, and -7 are thought to be the major effector caspases responsible for most of the proteolysis observed during the terminal or demolition phase of apoptosis. However, as we have seen here, caspase-3 also plays an important role in disseminating the caspase activation cascade and the relative roles of the effector caspases in the terminal phase of apoptosis remains unclear. It is also possible that Gzm B can target many of the same substrates that caspases cleave during apoptosis (7), as these proteases share a specificity for Asp in the P1 position of their substrate cleavage motifs.

To explore the relative importance of the effector caspase activation events initiated by Gzm B, we used cell-free extracts immunodepleted of the major effector caspases, caspase-3 and -7 (Fig. 4*A*). Note that immunodepletion of caspase-3 also had the effect of blocking activation of caspase-2, -6, and -9 (Fig. 3, *B* and *D*). Extracts induced to undergo caspase activation by addition of Gzm B displayed substantial DEVDase activity characteristic of the effector caspase-3 and -7 (Fig. 4*C*). Surprisingly, extracts depleted of caspase-7 still supported hydrolysis of the fluorogenic caspase substrate Ac-DEVD-AFC to the same degree as mock-depleted extracts (Fig. 4*C*). In marked contrast, caspase-3-depleted extracts had no measurable activity in the same assay suggesting that caspase-3 was responsible for essentially all of the DEVDase activity triggered by Gzm B (Fig. 4*C*). Immunoblots of caspase-3-depleted extracts confirmed that Gzm B induced full maturation of caspase-7, as expected, and suggested that caspase-7 could not functionally substitute for the loss of caspase-3 (Fig. 4*B*).

Global Analysis of Gzm B-induced Proteolysis Underscores the Importance of Caspase-3 as an Effector Caspase—The peptide hydrolysis data above suggested the possibility that

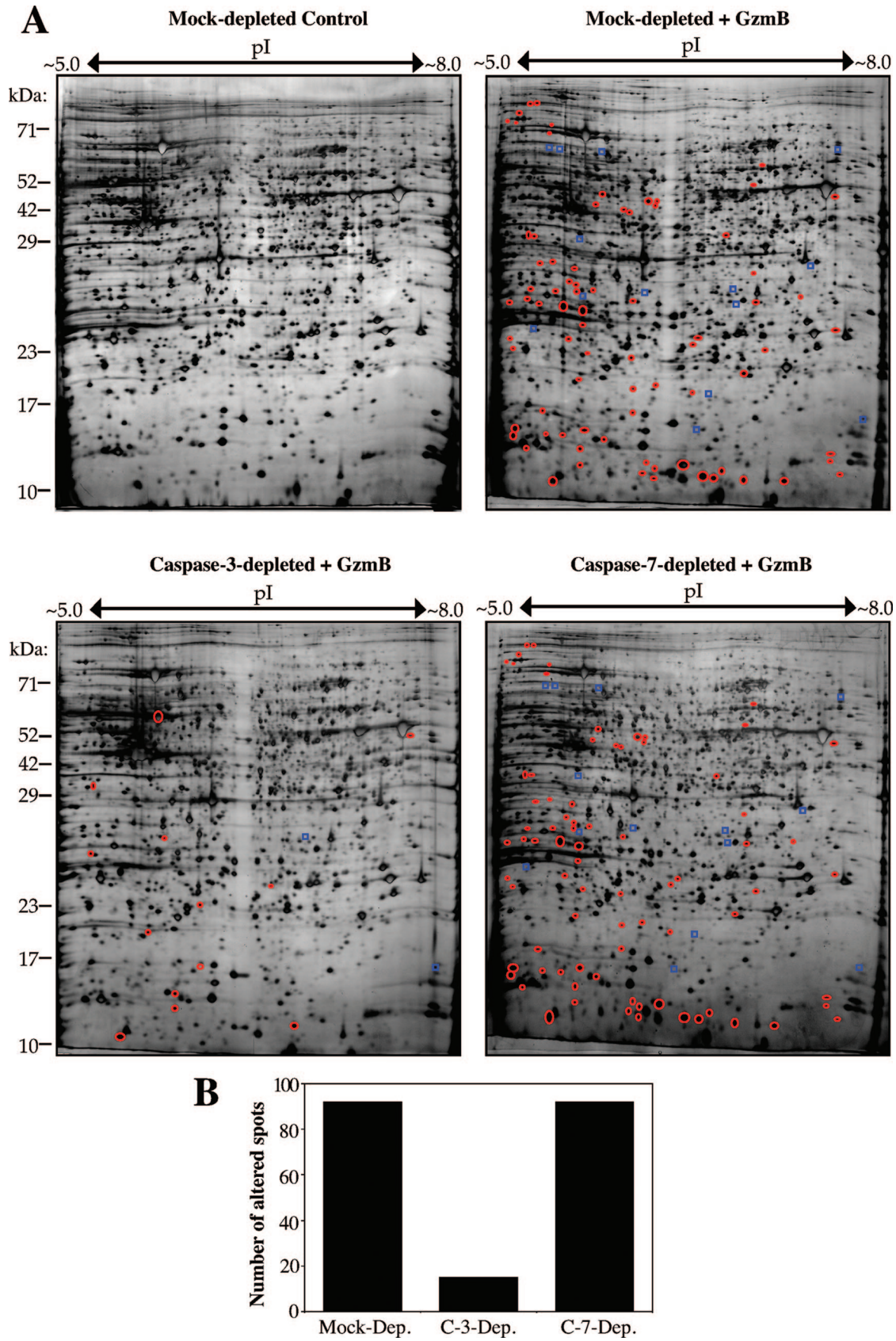

FIG. 5. **Depletion of caspase-3 abrogates the majority of Gzm B-induced proteolytic changes.** *A*, mock-depleted, caspase-3-depleted, or caspase-7-depleted cell-free extracts were incubated for 2 h at 37 °C in the presence or absence of human Gzm B (100 nM), as shown. Approximately 350 μg of protein from each reaction were subjected to isoelectric focusing on pH 5–8 IPGs, followed by 12% SDS-PAGE, as described under "Experimental Procedures." Gels were visualized by silver staining. Protein spots corresponding to novel proteolytic fragments not detected on the untreated control gel are encircled in *red*, whereas protein spots present in the control but not detected in Gzm B-treated samples are indicated by *blue squares*. *B*, histogram indicating the approximate total number of alterations detected in Gzm B-treated mock-depleted, caspase-3-depleted, and caspase-7-depleted extracts.

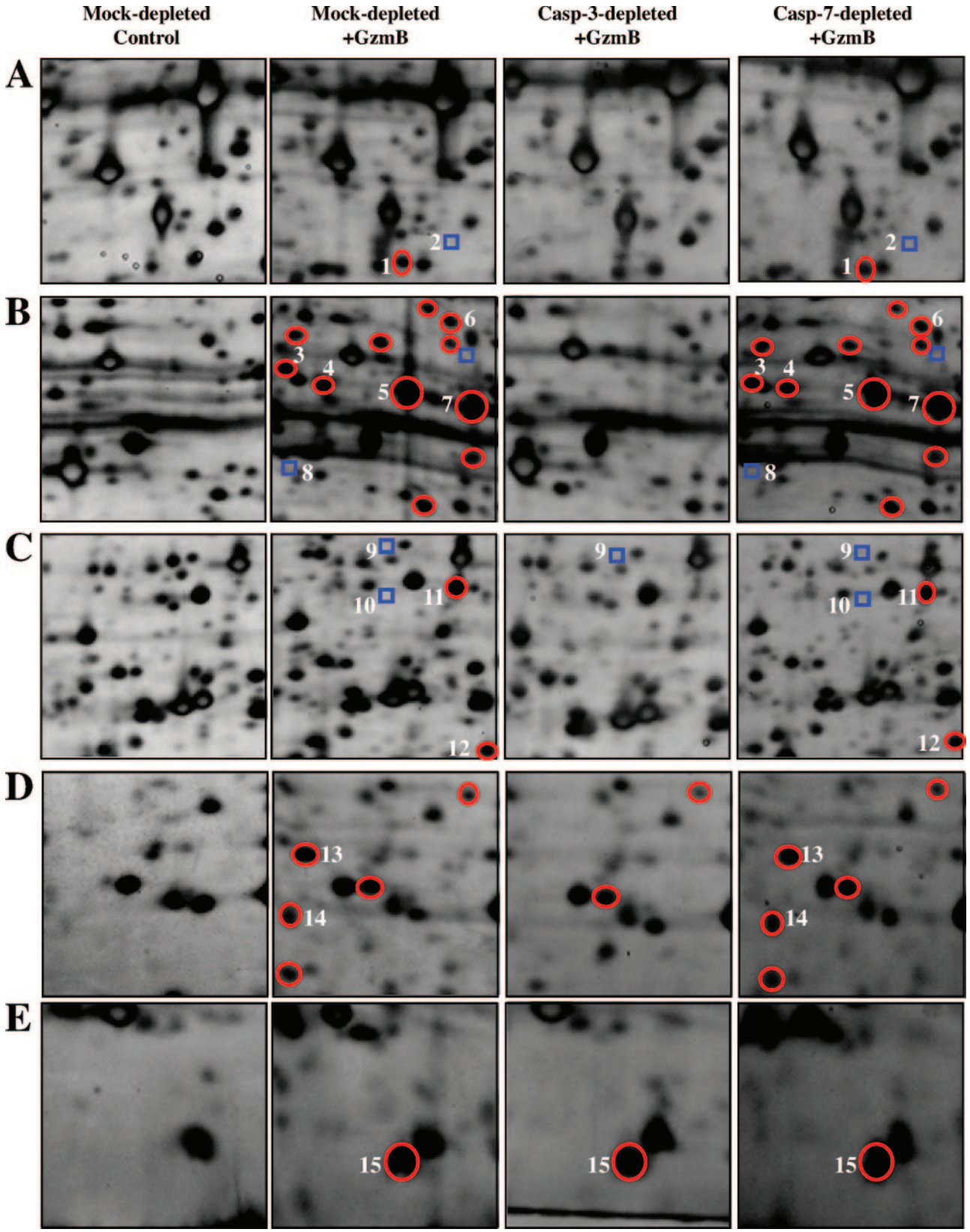

FIG. 6. **Identification of caspase-3-dependent proteomic alterations.** *A–D*, zoomed areas of two-dimensional gels highlight caspase-3-dependent protein spot alterations. Novel proteolytic fragments are encircled in *red*, whereas *blue squares* denote protein spots lost from the control treatment. The *numbers* denote caspase-3-dependent substrates that were identified by mass spectrometry. The identity of the numbered proteins is shown in Table I. *E*, an example of a non-caspase-3-dependent alteration that occurred in all Gzm B-treated extracts. The protein fragment encircled in *red* was identified as Tubulin-specific chaperone A.

caspase-3 was the major executioner activity initiated by Gzm B. To explore this possibility further, we ran two-dimensional SDS-PAGE gels to assess the number of Gzm B-induced alterations to the proteome seen in mock-depleted extracts *versus* extracts depleted of either caspase-3 or caspase-7 (Fig. 5*A*). We scored the appearance of new protein

TABLE I
Mass fingerprinting analysis of protein spots altered in extracts treated with Gzm B

Spot number	Protein identity	Accession number	Molecular mass/pI details	% coverage by mass spectrometry	Number of matched peptides
1	N-terminal acetyltransferase complex ARD1 subunit homolog	P41227	~25 kDa/5.5–5.6	42	15
2	Proteasome activator 28γ subunit	Q12930	29.506 kDa /5.7	48	14
3	PKC substrate, 80 kDa protein	P14314	~25 kDa/4.8	11	8
4	Actin	P02570	23.4 kDa/5.2	17	5
5	Actin	P02570	25.9 kDa/5.3	33	13
6	Alpha II spectrin	836669	39.311 kDa/5.27	24	17
7	Actin	P02570	23.5 kDa/5.8	27	11
8	Rho-GDP-dissociation inhibitor 2	P52566	23.031kDa/5.1	53	8
9	Caspase-3	P42574	31.594 kDa /6.1	48	15
10	Elongation initiation factor-4H	Q15056	27.385 kDa/6.7	32	10
11	Poly(rc)-binding protein 1 (heterogeneous nuclear ribonucleoprotein E1)	Q15365	~23 kDa/6.0	38	10
12	Guanidinoacetate *N*-methyltransferase	4503909	~23.3 kDa/6.2	60	21
13	Elongation factor 1 γ	P26641	~20.2 kDa/5.27	28	7
14	Phosphoribosyl pyrophosphate synthetase-associated protein 2	O60256	~16 kDa/5.5	26	8
15	Tubulin-specific chaperone A	O75347	~12. kDa/5.3	54	13
16	Mannose 6-phosphate receptor-binding protein	O60664	~44.3 kDa/5.9	36	13
17	Actin	P02570	~17 kDa/5.3	18	13
18	T-complex protein 1, β subunit	P78371	41 kDa/6.0	36	19
19	Rho-GDP-dissociation inhibitor 2	P52566	~18 kDa/6.8	45	9
20	Elongation initiation factor-5A	P10159	~16 kDa/5.6	51	8
21	Elongation initiation factor-5A	P10159	~15 kDa/6.1	40	8
22	Heterogeneous nuclear ribonucleoprotein K	Q07244	50.977 kDa/5.4	33	14
23	Heterogeneous nuclear ribonucleoprotein K	Q07244	50.977 kDa/5.4	55	28
24	Heterogeneous nuclear ribonucleoprotein K	Q07244	50.977 kDa/5.4	42	18

spots (fragments of cleaved proteins) in the Gzm B-treated samples, as well as the loss of protein spots upon treatment with Gzm B (Fig. 5*A*). Of approximately 2000 proteins examined, ~92 alterations to the protein spot patterns were detected by two-dimensional gel analysis upon treatment of extracts with Gzm B (Fig. 5*A*, *top right*). Consistent with the DEVD-AFC hydrolysis data, caspase-7-depleted extracts behaved essentially identical to mock-depleted extracts, with ~92 proteins being altered in response to addition of Gzm B (Fig. 5*A*, *bottom right*). In sharp contrast, immunodepletion of caspase-3 drastically reduced the number of protein spots that were altered after exposure to Gzm B from 92 to 15 (Fig. 5, *A*, *bottom left*, and *B*). These data suggest that the majority of alterations to the Jurkat cell proteome seen after treatment with granzyme B were caspase-3-dependent.

To identify some of the substrates that were cleaved in a caspase-3-dependent manner, a subset of the altered protein spots were excised from the two-dimensional gels and subjected to MALDI-TOF mass spectrometry (Fig. 6, *A–D*). The identity of these caspase-3-dependent proteolytic events is shown in Table I. This analysis revealed an essential role for caspase-3 in the cleavage of several known caspase substrates including actin, the proteasomal activator subunit PA28γ, components of the translational machinery (eIF4H, eEF1γ, and eIF5A), heterogeneous nuclear ribonucleoproteins E1 and K, and Rho-GDI2 (Table I and Fig. 6, *A–D*).

Of note, proteolysis of the tubulin folding cofactor, tubulin-specific chaperone A, was cleaved irrespective of whether caspase-3 or caspase-7 were present strongly suggesting that this protein is directly cleaved by Gzm B (Table I and Fig. 6*E*). More detailed analysis of similar, caspase-3-independent, Gzm B-induced proteolytic events revealed several novel Gzm B substrates, which will be described elsewhere.[2]

DISCUSSION

A Model for the Gzm B-initiated Caspase Cascade—Here we have undertaken a systematic analysis of the caspase activation events initiated by the CTL/NK protease, granzyme B. Previous studies have examined the ability of Gzm B (from a number of sources) to process and activate individual caspases, or a limited number of caspases, using diverse systems. Therefore it has been difficult to integrate the information from these disparate experimental settings into one model. In agreement with several previous studies, here we show that Gzm B is capable of direct processing of caspase-3, -7, -8, and -10 (Fig. 1; Refs. 13, 15, 16, and 19). Contrary to earlier reports, under conditions where adventitious propagation of the caspase cascade is blocked by the addition of Z-VAD-fmk, and under limiting concentrations of Gzm B, we fail to detect direct proteolysis of caspase-2, -6, or -9 by this granzyme (14, 17, 18). Using an immunodepletion approach we have shown that the latter caspases are engaged within the second tier of the Gzm B-intiated caspase cascade. As shown in Fig. 3, following its activation of Gzm B, caspase-3 plays a critical role in initiating a second tier of caspase activation events by processing caspase-2, -6, and -9.

Because Gzm B is also capable of directly processing and activating the BH-3-only molecule Bid, it is likely that a more complex cascade will pertain *in vivo* with Bid triggering the apoptosome cascade in parallel with the Gzm B-driven caspase cascade elucidated here (Fig. 3*D*; Ref. 27). In the apoptosome-initiated pathway, caspase-3 and -7 are activated simultaneously by caspase-9, after which the activation of caspase-2, -6, -8, and -10 requires caspase-3 (27). Thus caspase-3 plays a critical role in disseminating the caspase cascade irrespective of whether this initiated by Gzm B or via the Bid/apoptosome pathway.

A Novel Role for Caspase-3 in the Maturation of Caspase-8 and -10—We have found that although Gzm B directly processes caspase-8 and -10 between the large and small subunits, caspase-3 plays a critical role in removing the prodomains of these enzymes (Fig. 3). Although the current study has not addressed the question of whether the Gzm B-catalyzed intermediates of caspase-8 and -10 are functionally active, one possibility is that caspase-3-catalyzed removal of

[2] C. Adrain and S. J. Martin, manuscript in preparation.

the prodomain of these enzymes is required to facilitate their full enzymatic activity. Although further experimentation will be required to address this question, recent studies suggest that Gzm B-mediated processing does produce catalytically active caspase-8 (44).

Essential Role for Caspase-3 as the Major Gzm B-activated Executioner Enzyme—The current study has verified that at limiting levels of Gzm B, caspase-7 is inherently more sensitive to proteolysis than caspase-3 in human Jurkat cells (Fig. 1). Because caspase-7 is more sensitive to Gzm B-mediated activation, it was possible that the latter may play a more important role as a downstream mediator of the pro-apoptotic effects of Gzm B. However, subsequent analyses revealed that caspase-3 is the major DEVDase activity induced by Gzm B and plays a critical role in the proteolysis of numerous caspase substrates downstream. Indeed, we failed to detect any residual DEVDase activity in extracts devoid of caspase-3. It was beyond the scope of the current study to assess the relative functional importance of cleavage of the numerous caspase-3 substrates *versus* the small subset of substrates likely to be targeted by caspase-7. Superficially, however, these data strongly suggest that caspase-3 may be the major Gzm B-activated executioner caspase. It is certainly possible that some of the effects of caspase-3 may be indirect, given the requirement for caspase-3 in the processing of caspase-2, -6, and -9. However, whether in terms of propagation of the caspase cascade or at the level of direct substrate proteolysis, these data suggest an essential role for caspase-3 in disseminating the Gzm B-initiated death signal.

Relative Importance of Caspases Versus Bid in the Gzm B-initiated Death Pathway—In agreement with previous studies we have shown that, at limiting concentrations of Gzm B, human Bid is a better substrate for human Gzm B than caspase-3 (22, 23, 45). However, when murine Gzm B was added to cell-free extracts generated from murine J774 cells, as well as in human Jurkat cell extract, caspase-3 was clearly more efficiently cleaved than Bid. This raises the interesting possibility that murine and human Gzm B initiate apoptosis in subtly different ways. Several studies have implicated Bid as a key target of Gzm B during CTL/NK killing. Indeed, Bcl-2 has been shown to block Gzm B-induced killing in several experimental settings, suggesting that Gzm B may preferentially target the mitochondrial pathway, via Bid, rather than by directly activating caspases (23, 46). However, in contradiction to these observations, it has also been shown that Gzm B can still kill Bid-deficient or Bax/Bak-deficient cells, suggesting that Gzm B is capable of bypassing the requirement for mitochondrial involvement in caspase activation (31). Moreover, a recent study has suggested that Bid proteolysis during Gzm B killing is part of a caspase-3-initiated mitochondrial feedback loop and is not mediated by direct Gzm B cleavage (32). An explanation for some of these contradictory observations can almost certainly be explained by differences in the concentrations of Gzm B used between studies, differences in the mode of delivery to the target cell, and whether the Gzm B used was of murine or human origin. Interestingly, a recent report has highlighted that human caspase-7 cleaves the caspase substrate inhibitor of caspase-activated DNase only very poorly compared with the ability of its murine counterpart to cleave mouse inhibitor of caspase-activated DNase (47). Taken together with the present study, these data provide a cautionary note in relation to equating observations made within murine models to the human context.

Whether Gzm B accesses the caspase activation cascade directly or indirectly is likely to be heavily influenced by the concentration of this granzyme that is delivered by the CTL or NK cell. Irrespective of whether Bid or caspase-3 constitutes the direct preferential target of Gzm B, our data highlight the importance of caspase-3 as a critical executioner enzyme and key mediator of the apoptotic cascade during Gzm B killing. In summary, here we have shown that Gzm B triggers a two-tiered apoptotic cascade involving seven caspases and that caspase-3 plays a major role as a Gzm B-induced executioner enzyme.

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