Human and murine granzyme B exhibit divergent substrate preferences

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The cytotoxic lymphocyte protease granzyme B (GzmB) can promote apoptosis through direct processing and activation of members of the caspase family. GzmB can also cleave the BH3-only protein, BID, to promote caspase-independent mitochondrial permeabilization. Although human and mouse forms of GzmB exhibit extensive homology, these proteases diverge at residues predicted to influence substrate binding. We show that human and mouse GzmB exhibit radical differences in their ability to cleave BID, as well as several other key substrates, such as ICAD and caspase-8. Moreover, pharmacological inhibition of caspases clonogenically rescued human and mouse target cells from apoptosis initiated by mouse GzmB, but failed to do so in response to human GzmB. These data demonstrate that human and murine GzmB are distinct enzymes with different substrate preferences. Our observations also illustrate how subtle differences in enzyme structure can radically affect substrate selection.

Introduction

Granzyme B (GzmB) is an aspartic acid–directed protease that is contained within the specialized secretory granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (Masson et al., 1986; Masson and Tschopp, 1987; Trapani et al., 1999). Entry of GzmB, as well as other granzymes, into virally infected or transformed cells is facilitated by perforin, which is a pore-forming protein that is also contained within CTL/NK granules (Masson and Tschopp, 1985; Trapani and Smyth, 2002; Lieberman, 2003). Upon entry into target cells, GzmB can initiate apoptosis in the target through restricted proteolysis of substrate proteins (Darmon et al., 1995; Martin et al., 1996; Andrade et al., 1998; Barry et al., 2000; Browne et al., 2000; Sutton et al., 2000).

To date, several substrates for GzmB have been identified and, among these, the BH3-only protein BID has been implicated as playing a particularly important role (Heibein et al., 2000; Sutton et al., 2000; Alimonti et al., 2001; Pinkoski et al., 2001; Waterhouse et al., 2005). BID has been proposed to be the preferred substrate for GzmB (Pinkoski et al., 2001; Waterhouse et al., 2005, and BID proteolysis is thought to initiate apoptosis through activating Bax and/or Bak and promoting their oligomerization within the mitochondrial outer membrane. Oligomerization of Bax/Bak results in permeabilization of mitochondria and facilitates escape of intermembrane space proteins such as cytochrome c (Kuwana et al., 2002). Release of cytochrome c into the cytosol is especially relevant, as this protein acts to initiate the assembly of a complex between Apaf-1 and caspase-9, which results in the activation of the latter and precipitates a series of further caspase activation events culminating in the death of the cell (Li et al., 1997; Slee et al., 1999; Hill et al., 2004).

However, because GzmB can also directly process and activate caspases, such as caspase-3 and -8 (Darmon et al., 1995; Martin et al., 1996; Muzio et al., 1996; Medema et al., 1997; Adrain et al., 2005), this provides a more direct route to caspase-dependent cell death. Several other substrates for GzmB have been reported. Among these, ICAD (DFF45) is also thought to play an important role in cytotoxic lymphocyte killing, as this protein acts as an inhibitor of a DNase (CAD/DFF40) that becomes activated during many forms of apoptosis (Liu et al., 1997; Enari et al., 1998). Thus, it has been suggested that GzmB-mediated proteolysis of ICAD during CTL/NK-mediated killing can lead directly to CAD activation and DNA degradation (Thomas et al., 2000; Sharif-Askari et al., 2001).

Several studies have debated the relative importance of BID versus caspases and other GzmB substrates during CTL/NK-mediated killing (Sutton et al., 2000; Pinkoski et al., 2001; Alimonti et al., 2001; Metkar et al., 2003; Froelich et al., 2004; Waterhouse et al., 2005). One model proposes that BID proteolysis is the critical event in CTL/NK-mediated killing because BID (of human origin) appears to be more susceptible to GzmB-dependent proteolysis than caspases such as caspase-3 (Pinkoski et al., 2001). However, other studies have reported that...
BID proteolysis occurs secondary to caspase-3 activation in certain models (Metkar et al., 2003). One possible reason for these discrepant findings is that groups working in this area use GzmB preparations from either murine or human sources. Thus, some conclusions have been based on experiments where human GzmB has been used in conjunction with mouse cell lines or vice versa. This approach is predicated upon the assumption that human and murine GzmB have essentially identical substrate preferences, but this has not been established. However, this assumption does not take into account the divergence between human and mouse GzmB at residues that may affect substrate recognition or catalysis. Although the similarity between human and mouse GzmB is substantial, these are distinct proteases and are likely to display subtle, but important, differences in substrate selection. Similar considerations probably apply to many other conserved proteases, such as caspases.

We provide evidence that human and murine GzmB are distinct enzymes. Although both of these enzymes can cleave a similar cohort of protein substrates, we have found clear differences in their ability to cleave important substrate proteins such as BID, ICAD caspase-8, and several other substrates. These observations suggest that experiments that use GzmB (and possibly other granzymes) from one species cannot be readily generalized to other species. These observations have broad implications for our current understanding of the role of GzmB, as well as other granzymes, in CTL/NK-mediated killing.

Results

Titration of human and mouse GzmB using synthetic substrates

GzmB is a highly conserved protease, as the murine and human forms of this granzyme exhibit 69% identity (Fig. 1 A). However, although the amino acids that make up the catalytic triad are perfectly conserved between rat, mouse, and human GzmB (Fig. 1 A, residues highlighted in red), several of the residues that are predicted to influence substrate recognition exhibit significant divergence (Fig. 1 A, residues highlighted in blue). Because of the high degree of homology between human and murine GzmB, it is generally assumed that these enzymes cleave essentially the same cohort of substrates upon entry into target cells. Because of this, many investigators use these enzymes in cross-species experiments where mouse GzmB is used to kill human target cells or vice versa. However, the divergence seen in residues involved in substrate recognition suggests that human and mouse GzmB may not behave in an identical manner toward the same protein substrates.

To explore this issue, we generated recombinant human and mouse GzmB in Pichia pastoris and active site–titrated these enzymes using the synthetic substrate peptide Ac-IETD-AFC and the viral serpin CrmA (Fig. 1 B). Using this assay, we chose concentrations of the human and murine enzymes that displayed equivalent rates of IETD-AFC hydrolysis and used these concentrations of GzmB for subsequent experiments (Fig. 1, C and D). We then compared hGzmB and mGzmB for their ability to process and activate effector caspases within cell-free extracts prepared from either human (Jurkat) or mouse (J774) cell lines. To permit direct comparison, cell-free extract preparations were adjusted to the same final protein concentration (10 mg/ml). Effector caspase activation was assessed by measuring the hydrolysis of Ac-DEVD-AFC, which is a peptide that is cleaved efficiently by caspase-3 and -7, but not by GzmB (Sarin et al., 1996).

As illustrated in Fig. 1 E, addition of 100 nM of either human or mouse GzmB to cell-free extracts of human origin provoked similar rates of DEVD-AFC hydrolysis, suggesting that these enzymes activated effector caspases within the extracts with equal efficiency. Note that neither granzyme hydrolyzed DEVD-AFC when cell-free extract was omitted from the assay (Fig. 1 E, left). Essentially identical results were observed when cell-free extracts generated from mouse J774 cells were used in the same assay (Fig. 1 E, right). These data suggest that human and mouse GzmB activate effector caspases (whether of human or murine origin) with a similar efficiency.

BID is a poor substrate for mouse GzmB

As outlined earlier, it has been suggested that BID is the preferred substrate for GzmB (Sutton et al., 2000; Pinkoski et al., 2001; Waterhouse et al., 2005). Proteolysis of BID by GzmB results in the exposure of a myristoylation signal sequence that, upon myristoylation at this site, promotes translocation of BID to mitochondria to induce oligomerization of Bax and Bak on the mitochondrial outer membrane (Zha et al., 2000). Because cleaved BID is a potent trigger for mitochondrial cytochrome c release, proteolysis of this BH3-only protein may well be sufficient to ensure death of a target cell upon CTL attack. Thus, we were interested in determining whether the human and mouse forms of GzmB cleaved BID with similar efficiency. We also compared hGzmB and mGzmB in terms of their ability to proteolytically process caspase-3, -7, and -8, all of which are well established as substrates for hGzmB (Adrain et al., 2005). As a source of human BID and other human substrate proteins, we used cell-free extracts derived from Jurkat cells (Martin et al., 1996; Slee et al., 1999). As a source of murine substrates, similar cell-free extracts were generated from the mouse macrophage line, J774. To exclude complex outcomes resulting from activation of caspases within the extracts caused by the addition of exogenous GzmB, we also included 10 μM zVADfmk in all reactions to suppress endogenous caspase activity. Note that this poly(ADP-ribose)polymerase inhibitor does not inhibit GzmB activity (Adrain et al., 2005; Adrain et al., 2006).

As Fig. 2 (A and B) illustrates, addition of 100 nM hGzmB to the cell-free extracts led to efficient proteolysis of BID of human and murine origin, and to slightly less efficient proteolysis of caspase-3 and -7 in the same extracts. In contrast, although hGzmB was found to proteolytically process human caspase-8 (Fig. 2 A), it failed to cleave the mouse counterpart of this caspase (Fig. 2 B). mCaspase-8 was processed upon addition of cytochrome c and dATP to J774 extracts to activate the apoptosome pathway to caspase activation (Fig. S1, http://www.jcb.org/cgi/content/full/jcb.200612025/DC1), confirming that the antibody used to detect mcaspase-8 was, indeed, capable of detecting the processed form of this enzyme. Thus, mcaspase-8 does not appear to be a substrate for hGzmB.
Rather surprisingly, mGzmB failed to cleave BID of either species under conditions where hGzmB clearly did so (Fig. 2, A and B). Importantly, mGzmB cleaved both human and mouse caspase-3 and -7 very efficiently under the same conditions (Fig. 2, A and B), thereby precluding the possibility that mGzmB was not active in these experiments. In addition to the differences observed in respect to BID proteolysis, it can also be seen that mGzmB failed to cleave caspase-8 from either species (Fig. 2).

We also performed titrations of human and mouse GzmB in Jurkat and J774 cell-free extracts and, again, observed marked differences in the ability of these enzymes to cleave BID and caspase-8 (Fig. 2, C and D). We also assessed proteolysis of nucleophosmin/B23 in these assays, as this protein has recently been reported to be a substrate for hGzmB (Ulanet et al., 2003). However, whereas hGzmB efficiently cleaved nucleophosmin of human origin, mGzmB cleaved this protein very inefficiently under the same conditions (Fig. 2 C). Moreover, neither human nor mouse GzmB cleaved nucleophosmin within J774 cell-free extracts (Fig. 2 D). Thus, although human and mouse GzmB can proteolytically process caspase-3 and -7 (whether of mouse or human origin), these granzymes exhibited distinct differences in respect to their ability to cleave BID, caspase-8, and nucleophosmin.

To exclude the possibility that the results we observed could be indirect effects, e.g., caused by activation of caspases...
within the cell-free extracts, we repeated these experiments using purified recombinant human and mouse GzmB. As Fig. 3 illustrates, both human and mouse BID were cleaved efficiently by hGzmB. As a control, we also compared the efficiency of proteolysis of recombinant pro–caspase-7, and this was cleaved by hGzmB with comparable efficiency to that of BID (Fig. 3). However, in complete agreement with the results generated using cell-free extracts, although mGzmB cleaved caspase-7 with an essentially identical efficiency to hGzmB, it failed to exert any detectable proteolytic activity toward BID from either species (Fig. 3). These data provide robust evidence that human and mouse GzmB display divergent activity toward BID, but similar activity toward caspase-7.

Differential proteolysis of ICAD by human and mouse GzmB

We then extended our analysis to several other proteins that have been reported to be substrates for GzmB. ICAD, the inhibitory subunit for the CAD deoxyribonuclease that plays a role in apoptosis-associated DNA hydrolysis, has also been reported to be a direct substrate for GzmB (Thomas et al., 2000; Sharif-Askari et al., 2001). Consistent with this, hGzmB cleaved ICAD efficiently in Jurkat cell-free extracts (Fig. 4 A). However, mGzmB failed to cleave human ICAD under the conditions where human ICAD was readilycleaved by hGzmB (Fig. 4 B). We also assessed several additional GzmB substrates in these assays (cochaperone p23, CD2-associated protein, and α-tubulin), and in each case found substantial differences between the activity of human and mouse GzmB toward these substrates (Fig. 4, A and B).

Collectively, these observations suggest that human and mouse GzmB are quite distinct enzymes in terms of their activity toward the same protein substrates. This raises the possibility that mGzmB may rely predominantly upon caspase activation to kill target cells.

Inhibition of caspases clonogenically rescued cells from mGzmB, but not from hGzmB

From the preceding experiments, it was apparent that human and mouse BID are poor substrates for mGzmB, whereas both human and murine caspases -3 and -7 are readily cleaved by this granzyme (Figs. 2 and 3). In contrast, human GzmB cleaved BID of either species very efficiently (Figs. 2 and 3). An important
Implication of these observations is that murine GzmB may be unable to activate the BID pathway to kill target cells and may rely predominantly on direct activation of caspases. This suggests that inhibition of caspases may clonogenically rescue cells from mouse, but not human, GzmB.

To test this possibility, we loaded human CEM cells with either human or mouse GzmB using the pore-forming protein streptolysin O (SLO). As shown in Fig. 5, loading of CEM cells with human or mouse GzmB led to very rapid death (within 6 h) with all of the typical features of apoptosis. Inclusion of the caspase inhibitor zVADfmk in these assays transiently delayed apoptosis induced by hGzmB (Fig. 5). However, within 48–72 h, cells treated with hGzmB had essentially all died, irrespective of the presence of the caspase-inhibitory peptide (Fig. 5).
In contrast, when the same cells were loaded with mGzmB at concentrations that achieved >95% cell death within 6 h in the absence of zVADfmk (Fig. 5), these cells were substantially protected when the caspase inhibitor was included in the assay. Moreover, the protection afforded by zVADfmk was long-term, as these cells proliferated upon subsequent culture (Fig. 5 D).

Essentially identical results were also observed using human Jurkat cells in the same assay (Fig. 6, A and B), and these cells could also establish colonies when plated in soft agar (Fig. 6, C and D).

Although caspase inhibition selectively maintained the clonogenic potential of two different human cell lines treated with mGzmB, it remained possible that this granzyme could kill mouse cells in a caspase-independent manner. Thus, we performed the reciprocal experiment using mouse J774 cells, and in this case we used a different polycaspase inhibitor, Q-VD-Oph (Caserta et al., 2003). As Fig. 7 shows, whereas J774 cells were readily killed by both GzmB species, inhibition of caspases only restored clonogenic potential in cells that were treated with mGzmB. Furthermore, under conditions where mGzmB efficiently killed J774 cells, no detectable processing of BID was observed, whereas caspase-3 processing was readily detected (Fig. 7 C). In contrast, hGzmB cleaved both BID and caspase-3 in the same cells (Fig. 7 C).

Using MCF-7 cells deficient in caspase-3, we also explored whether the absence of this caspase was sufficient, on its own, to confer protection against mGzmB. For comparison, we also used MCF-7 cells reconstituted with caspase-3 by stable transfection of a CASP-3 expression plasmid (Janicke et al., 1998). However, although the absence of caspase-3 did delay the onset of GzmB-initiated cell death, no long-term protection was seen in the absence of this caspase (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200612025/DC1). This suggests that GzmB-mediated processing of other caspases, such as caspase-7 (Figs. 2 and 3), is probably sufficient to kill cells in the absence of caspase-3, and that inhibition of both proteases is necessary to achieve cloning protection from mGzmB.

Collectively, the data presented in this study suggests that caspases are essential for apoptosis initiated by mouse, but not human, GzmB, and underscores the distinct substrate preferences of these closely related proteases. Although BID and ICAD were cleaved readily by human GzmB, neither protein was efficiently cleaved by the murine form of this protease. These results suggest that an apparently minor divergence in primary amino acid sequence between two closely related enzymes can lead to functionally important shifts in substrate selection. A broader implication of these observations is that other proteases that have diverged more substantially between mouse and man, such as caspases, are also likely to display different substrate preferences that may profoundly affect their mechanism of action.

Discussion

We have shown that human and mouse GzmB behave as distinct proteases and share only partial overlap in their substrate range. In particular, whereas hGzmB efficiently cleaved the BH3-only protein BID, mGzmB failed to do so. Similarly, hGzmB promoted efficient proteolysis of the human forms of ICAD and caspase-8, whereas mGzmB failed to cleave these proteins. Interestingly, neither human nor mouse GzmB cleaved murine ICAD or murine caspase-8 under the conditions used in this study. Several other examples of divergent substrate preferences between human and murine GzmB were found. Collectively, these data demonstrate that human and mouse GzmB exhibit clear differences in the cohort of proteins that these proteases target upon entry into target cells. These observations are also supported by a study from Kaiserman et al. (2006) that was published during revision of our study, as these authors also found similar functional divergence between human and mouse GzmB.

There has been much debate concerning the relative importance of the substrates cleaved by GzmB (Pinkoski et al., 2001; Metkar et al., 2003; Froelich et al., 2004; Waterhouse et al., 2005). Although some have suggested that BID may be the most relevant GzmB substrate during CTL attack, others
have suggested that BID proteolysis is indirect and is mediated by caspase-3, which is also activated by GzmB (Metkar et al., 2003). The contention that BID is a preferential substrate for GzmB is supported by studies that have shown that Bcl-2 can block GzmB-initiated apoptosis, whereas caspase inhibitors fail to do so (Pinkoski et al., 2001). The latter observations would support an upstream role for BID, independent of caspase activity, in promoting mitochondrial outer membrane permeabilization as a result of Bax/Bak activation. In this scenario, inhibition of caspase activity would fail to block mitochondrial outer membrane permeabilization, and cells would die in a caspase-independent manner. Bcl-2 would be expected to block...
death in this context through antagonizing the effects of BID on mitochondria.

The observation that BID is a poor substrate for mGzmB suggests that this protease does not efficiently activate the BID pathway to cell death in the mouse. Consistent with this, although inhibition of caspase activity failed to clonogenically rescue cells from hGzmB, clonogenic rescue was readily seen when mGzmB was used in the same context. This was true irrespective of whether human or mouse target cells were used. These observations have important implications for studies where murine granzymes were evaluated in human cell lines and vice versa. For example, recent studies have used BID-null mouse embryonic fibroblasts in conjunction with hGzmB to conclude that BID is an essential target for GzmB in this system (Waterhouse et al., 2005). Our observations suggest that mGzmB kills in a predominantly caspase-dependent and BID-independent manner, whereas the reverse could be true for hGzmB. However, an alternative interpretation is that BID is not important for GzmB-mediated cell death in either organism. Thus, conclusions based on mixing human enzymes with mouse substrates (or the reverse) should be interpreted with caution.

The implications of our findings also extend to other related proteases, such as caspasps, where it is often assumed that human and murine orthologues perform essentially identical functions. However, the sequence identity between human and murine caspase orthologues is typically much lower (on average 40%) than seen with GzmB. This makes it even more likely that functionally important differences in the substrate range of human and mouse caspase orthologues will emerge upon examination of this issue. Indeed, there is already one clear example of sequence divergence between murine and human caspsps that has major functional consequences. Whereas murine caspase-12 appears to be a functional enzyme (Kalai et al., 2003), human caspase-12 has acquired substitutions that render this caspase inactive (Fischer et al., 2002). Although this is clearly an extreme example, it is likely that less obvious examples of functional divergence between caspase orthologues will emerge, as we have seen for GzmB, when this is explored.

In summary, our data demonstrate that human and murine GzmB behave as distinct enzymes with divergent substrate preferences. These observations illustrate how subtle differences in enzyme structure can radically affect substrate selection.

Materials and methods

Materials

The following antibodies were used: anti–caspase-3, anti–human caspase-7, anti–XIAP, and anti–human BID (BD Biosciences); anti–mouse caspase-7 (Millipore); anti–mouse BID (R&D Systems); anti–human caspase-8 (OncoGene); anti–mouse caspase-8, (Alexis); anti–a-tubulin and anti–actin (ICN, UK); anti–p23 (Affinity BioReagents); anti–nucleophosmin (Invitrogen); anti–CD2-associated protein (Santa Cruz Biotechnology, Inc.); anti–DFF45 (Calbiochem and Biosource). The peptides zVADfmk and Ac-DEVD-AFC were purchased from Bachem, IETD-AFC was purchased from Alexis, Noble agar was purchased from BD Biosciences, and SLO was purchased from Aalto Bio Reagents. Vivaspin 0.5 ml microcentrifuge concentrators were purchased from Sartorius. Unless otherwise indicated, all other reagents were purchased from Sigma-Aldrich.

Production of human and mouse GzmB in P. pastoris

The cDNA encoding the mature form of mouse GzmB was cloned into the expression vector pPIC6a (Invitrogen). P. pastoris X-33 cells were transformed with pPIC6a.mGzmB via electroporation and were put through two rounds of selection in 900 μg/ml blasticidin, resulting in X-33 clones stably harboring mouse GzmB expression plasmid. P. pastoris yeast clones stably harboring a human GzmB expression plasmid were provided by W. Wels (Chemotherapeutisches Forschungsinstitut Georg-Speyer-Haus, Frankfurt, Germany). Yeast clones expressing hGzmB were grown in 1-liter cultures for 3 d at 25°C, whereas clones expressing mGzmB were grown in 2-liter cultures for 4 d at 25°C. In both cases, GzmB was purified from culture supernatants using nickel affinity chromatography, followed by extensive washing in buffer containing 5 mM imidazole. Purified GzmB was eluted with 500 mM imidazole and washed extensively in PBS, pH 7.2, followed by concentration in microcentrifuge units (Sartorius).
Expression and purification of recombinant proteins in bacteria

The pET15b expression plasmid encoding full-length human BID was provided by X.-M. Yin (University of Pittsburgh School of Medicine, Pittsburgh, PA), the pET23dwtHis expression plasmid containing full-length mouse BID was provided by X. Wang (University of Texas Southwestern Medical Center, Dallas, TX), and G.S. Salvesen (The Burnham Institute of Medical Research, Pittsburgh, PA) provided the pET23b expression plasmid containing full-length human caspase-7. Expression plasmids were transformed into the bacterial E. coli strain BL21 (DE3) and were expressed and affinity purified as previously described (Hill et al., 2004), with the exception that caspase-7 was induced for 40 min to minimize the production of the processed form of this protease. Full-length CrmA was cloned into the expression plasmid pGEX4T2K2 (GE Healthcare) in frame with the GST coding sequence at the N terminus. GST-CrmA was expressed in E. coli DH5α and affinity purified using glutathione-Sepharose as previously described (Slee et al., 1999).

Active site titration of recombinant granzymes

1 μM GSTCrmA was activated by incubation with 500 μM dithiothreitol for 10 min at 37°C. Active CrmA was then incubated with human or mouse GzmB for 10 min at 37°C. Residual GzmB activity was then determined by diluting samples into PBS containing 500 μM Ac-IETD-AFC and measuring hydrolysis of the latter peptide by fluorimetry, as described in Fluorometric assays.

Kinetic analysis of substrate proteolysis by granzymes

Typically, 3–4 μg of recombinant human or mouse BID was incubated for 2–3 h at 37°C with varying concentrations of human or mouse GzmB in 10 μl reaction volumes. Human recombinant pro-caspase-7 was treated in an identical manner, but with 10 μM zVADfmk added to inhibit caspase autoprocessing. Reaction products were separated by SDS-PAGE and visualized by Coomassie blue staining. Densitometry was performed using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/) and catalytic rates were calculated using these data.

Cell-free reactions

Cell-free cytosolic S-15 extracts were generated from Jurkat and J774 cells, as previously described (Martin et al., 1996; Slee et al., 1999). Typically, human and murine extracts were normalized to 10 mg/ml total protein content, and then diluted twofold in WCEB (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotonin). GzmB and zVADfmk were added to the reactions at final concentrations of 6–100 nM and 10 μM, respectively. Reactions were incubated for 1–2 h at 37°C to facilitate GzmB-mediated proteolysis. Cell-free reactions were then assessed by immunoblots or fluorometric assay.

Fluorometric assays

Reactions containing Jurkat or J774 cell-free extracts (normalized for total protein content) were typically assayed in a final volume of 10 μl. After a 30-min incubation at 37°C, 2.5 μl samples were diluted to a final volume of 100 μl in WCEB containing 50 μM Ac-DEVD-AFC. To determine the active site titration of recombinant GzmB, protein was diluted in 30 μl PBS containing 500 μM Ac-IETD-AFC. All samples were measured in an automated fluorimeter (Spectrafluor Plus; TECAN) at wavelengths of 430 (excitation) and 535 nm (emission).

Clonogenic assays

For clonogenic assays on Jurkat and J774 cells, a 3-mm bottom agar layer was poured on 6-cm culture dishes containing RPMI with 20% fetal calf serum, 1% l-Glutamine, 2 mg/ml sodium bicarbonate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotonin. GzmB and zVADfmk were added to the reactions at final concentrations of 6–100 nM and 10 μM, respectively. Reactions were diluted twofold in WCEB containing 500 μM Ac-IETD-AFC. All samples were measured in an automated fluorimeter (Spectrafluor Plus; TECAN) at wavelengths of 430 (excitation) and 535 nm (emission).

Online supplemental material

Fig. 1 shows the cytochrome c/dATP-induced processing of caspase-8 in mouse J774 cell-free extracts. Fig. S2 shows that caspase-3 deficiency is not sufficient to afford clonogenic rescue from human or mouse GzmB. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200612025/DC1.

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