

Identification of α -tubulin as a granzyme B substrate during CTL-mediated apoptosis

Ing Swie Goping¹, Tracy Sawchuk¹, D. Alan Underhill² and R. Chris Bleackley^{1,*}

¹Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7

²Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7

*Author for correspondence (e-mail: chris.bleackley@ualberta.ca)

Accepted 14 November 2005

Journal of Cell Science 119, 858-865 Published by The Company of Biologists 2006
doi:10.1242/jcs.02791

Summary

Cytotoxic lymphocytes induce target cell apoptosis via two major pathways: Fas/FasL and granule exocytosis. The latter pathway has largely been defined by the roles of the pore-forming protein perforin and by the serine proteinases granzymes A and B. Upon entry into target cells, the granzymes cleave substrates that ultimately result in cell death. To gain further insight into granzyme B function, we have identified novel substrates. SDS-PAGE analysis of S100 cell lysates identified a 51 kDa protein that was cleaved by granzyme B. Mass spectrometry analysis revealed that this fragment was the microtubule protein, α -tubulin, which was confirmed by western blotting. In addition, two-dimensional gel analysis showed that the truncated form of α -tubulin had a more basic isoelectric point than the full-length molecule, suggesting that

granzyme B removed the acidic C-terminus. Site-directed mutagenesis within this region of α -tubulin revealed the granzyme B recognition site, which is conserved in a subset of α -tubulin isoforms. Significantly, we showed that α -tubulin was cleaved in target cells undergoing apoptosis as induced by cytotoxic T lymphocytes. Therefore, in addition to its role in the activation of mitochondria during apoptosis, these results suggest a role for granzyme B in the dismantling of the cytoskeleton.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/119/5/858/DC1>

Key words: Apoptosis, Tubulin, Cytotoxic lymphocyte

Introduction

Cytotoxic lymphocytes (CLs) are important effector cells of the immune system. CLs, which include both Natural killer (NK) cells and the cytotoxic T lymphocytes (CTLs) function through signals delivered by direct contact with their target. Unlike complement and antibody-mediated cell clearance, which largely involve plasma membrane destruction, CLs activate destruction by initiating the apoptotic program of the target cell (for reviews, see Barry and Bleackley, 2002; Russell and Ley, 2002; Waterhouse et al., 2004). This is accomplished through two main mechanisms. In one, the Fas/FasL pathway, CLs trigger a receptor-mediated extrinsic apoptotic program in the target cell. Studies in mouse models have shown that this pathway predominately regulates lymphocyte homeostasis (Nagata and Golstein, 1995). A second mechanism, the granular pathway, involves CL-mediated delivery of toxic molecules to the target cell (Henkart, 1985). This pathway is characterized by cytolytic granule polarization (Yannelli et al., 1986) and exocytosis of granule contents into the tight synaptic space that is formed between the CL and target cell (Stinchcombe et al., 2001). Molecules that are released from the granules are taken up by the target cell and trigger apoptosis (Shi et al., 1992). The granular pathway is necessary for the elimination of some classes of virus-infected cells (Trapani and Sutton, 2003) and tumor cells (Kagi et al., 1994). In addition to its beneficial functions, this pathway also contributes to immunopathology, as it has been implicated in autoimmune diseases, allo-graft rejection, and graft versus host disease

(GvHD). Thus, modulation of the granular pathway has important implications for therapeutic use, because augmentation of granular killing would enhance tumor control, whereas downregulation of the response would manage autoimmune disease or GvHD. It is therefore important to more fully elucidate the granular pathway and identify the many steps that culminate in target cell death.

The cytotoxic granule contains many components that contribute to the death of the target cell. The pore-forming protein, perforin, is required for granular-mediated apoptosis (Kagi et al., 1994; Kojima et al., 1994; Lowin et al., 1994), and the granzymes also play a major role; the most abundant of which are granzymes A and B. These proteolytic enzymes activate parallel pathways within the target cell (Lieberman, 2003). Initially, granzyme B (grB) was the most extensively studied of the granzymes (Lord et al., 2003; Roberts et al., 2003) and was found to be a serine protease with a unique specificity for aspartic acid residues similar to the apoptotic caspases. As such, it was proposed to function as an initiator protease of the caspase cascade. In support of this idea, grB cleaves the effector caspase, caspase 3, in vitro (Darmon et al., 1995; Martin et al., 1996; Van de Craen et al., 1997). In cells, however, grB-cleaved caspase 3 is catalytically inert, because its enzymatic activity is attenuated by inhibitor of apoptosis proteins (IAPs) (Goping et al., 2003; Sutton et al., 2003). The de-repression of IAPs occurs in turn by grB-directed cleavage of the proapoptotic protein Bid (Barry et al., 2000), which translocates to the mitochondria and initiates events that lead

to efflux of cytochrome c (Heibein et al., 2000; Sutton et al., 2000), Smac/DIABLO and Omi/HtrA2 (Goping et al., 2003; Sutton et al., 2003). The latter two proteins remove the caspase 3 block, resulting in caspase 3 autoprocessing and eventual cell death (Goping et al., 2003; Sutton et al., 2003).

In addition to the caspase-dependent grB functions described above, cell death can be elicited in the presence of caspase inhibitors (Trapani et al., 1998), making it clear there are additional pathways that contribute to the apoptotic process. In order to determine the mechanism(s) involved, many laboratories have assessed the role of non-caspase grB targets, although no definitive pathway has yet emerged. For example, grB has been shown to bypass the need for caspase 3-dependent activation of the apoptotic DNase, CAD, through direct cleavage of its inhibitor, ICAD (Sharif-Askari et al., 2001; Thomas et al., 2000; Wolf et al., 1999). Nevertheless, it has been suggested that grB is not the major inactivator of ICAD (Wolf et al., 1999). Alternatively, caspase-independent DNA fragmentation could result if grB orchestrated the release of not only caspase-activating proteins such as cytochrome c, Smac/DIABLO and Omi/HtrA2, but also the nucleases endonuclease G (Li et al., 2001), and apoptosis-initiating factor (AIF) (Susin et al., 1999) from mitochondria. Unfortunately, it is not clear whether these nucleases are released into the cytosol of grB-treated cells. Despite these alternative mechanisms, we in fact find that grB-mediated DNA fragmentation is highly dependent on caspases and that cells probably die as a result of subsequent membrane damage that does not require caspases. As a result, the caspase-independent pathways of grB remain to be identified.

In order to map the numerous pathways that are activated by grB, it is important to look for relevant grB substrates. Other grB targets that have already been documented include nuclear, cytosolic and extracellular proteins (Adrain et al., 2005; Bredemeyer et al., 2004; Russell and Ley, 2002; Trapani and Sutton, 2003); although the relevance of the cleavage of most of these proteins is unclear. The identification of further grB substrates and, more importantly, their functional contributions to apoptosis, will be essential to gain a more complete understanding of the biological role of grB. To that end, we have analyzed proteins derived from an S100 cytosolic extract and show that the microtubule protein, α -tubulin, is a substrate. Previously, Bredemeyer et al. have shown that grB could cleave α -tubulin (Bredemeyer et al., 2004). Our data confirm that result and extend the study to identify the grB-cleavage site at position D438. Most importantly, we establish the physiologic relevance by the demonstration that cleaved α -tubulin is also present in target cells that are induced to undergo apoptosis by CTL. The cleaved α -tubulin was not associated with the assembled microtubule network, implicating an involvement in cytoskeletal collapse during apoptosis. The possible implications of α -tubulin cleavage during CTL-mediated apoptosis are discussed.

Results

HeLa cytosolic extract contains a 51 kDa protein that is cleaved by granzyme B

In order to identify substrates of grB we performed *in vitro* grB cleavage assays of proteins from cell lysates, and analyzed the resulting protein profiles. HeLa S100 extracts were treated with increasing amounts of purified grB. The lysates were subjected

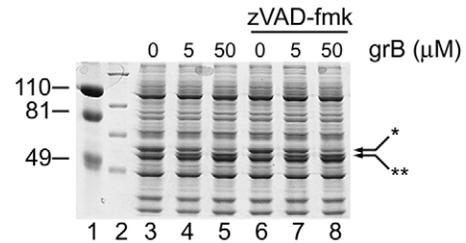


Fig. 1. Coomassie-Blue-stained gel of HeLa S100 extracts. 30 μ g HeLa S100 extract were left untreated (lane 3) or treated with 5 μ M and 50 μ M purified grB in a 10 μ l reaction volume (lanes 4-5, 7-8). 100 μ M zVAD-fmk was added to lanes 6-8. Protein products were separated by a 12% SDS-PAGE and visualized by staining with Coomassie Blue R250. Positions of molecular size markers (in kDa) are indicated on the left.

to SDS-PAGE and proteins were visualized by staining with Coomassie Blue. A prominent protein band of 51 kDa that was present in HeLa cytosolic extracts, diminished in intensity upon incubation with grB (Fig. 1, compare lane 3 with 4-5, marked with *). In addition, a protein fragment of 49 kDa that was not detected in untreated samples, became visible upon treatment (Fig. 1, compare lane 3 with 4-5, marked with **). The cleavage was probably due to grB activity and not caspase activation, as this pattern was similar in cell lysates treated in the presence and absence of the general caspase inhibitor zVAD-fmk (Fig. 1, compare lanes 3-5 with 6-8). Unlike caspase activity, grB remains enzymatically active in the presence of zVAD-fmk, which was evident by its ability to cleave its substrate pro-caspase 3 to the p20 fragment (data not shown, and Fig. 2B).

The 51 kDa grB-cleaved protein is identified as α -tubulin. We reasoned that the 49 kDa fragment that appeared in the grB-treated samples was a cleavage product of the 51 kDa protein band that was present in untreated S100 samples. Therefore both protein bands were excised from a preparative SDS-PAGE and subjected to trypsin digestion and mass spectrometry analysis. The analysis provided five tryptic fragments that were common to both the 51 kDa and 49 kDa samples, all of which corresponded to α -tubulin (Fig. 2A). Cleavage of α -tubulin was confirmed by western blot analysis of grB-treated S100 lysates, (Fig. 2B, upper panel). Similar to the pattern of the Coomassie-Blue-stained protein identified in Fig. 1, α -tubulin derived from HeLa S100 extracts was cleaved by grB in a caspase-independent manner. Furthermore, α -tubulin derived from Jurkat S100 extracts, was also found to be cleaved by grB in a caspase-independent manner (Fig. 2B upper panel, compare lanes 1-6 with 7-12). To ensure that the zVAD-fmk did indeed inhibit caspase activation, the caspase 3 maturation profile was analyzed on a western blot. The caspase-dependent maturation of p20 to p19 (Atkinson et al., 1998), was abolished in the presence of zVAD-fmk (Fig. 2B lower, compare lane 3 with 6, and lane 9 with 12). GrB, however, remained enzymatically active in the presence of zVAD-fmk, as it was still able to cleave its target, the full-length pro-caspase 3, from its unprocessed 32 kDa form to the p20 fragment (Fig. 2B lower, lanes 6 and 12).

A

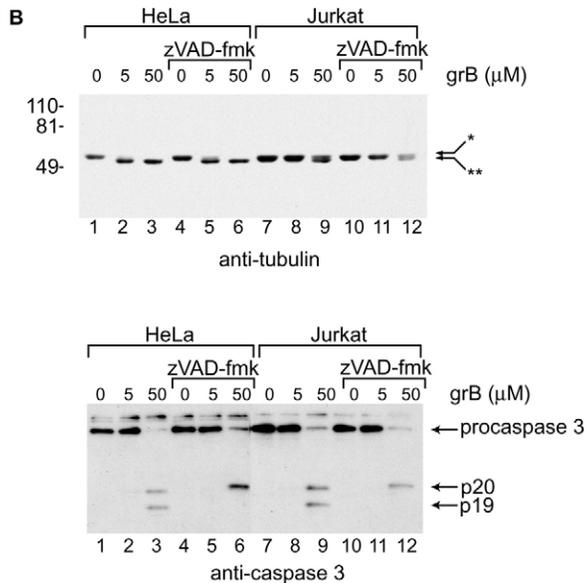
```

1  MRECISIHVG QAGVQIGNAC WELYCLEHGI QPDGQMPSEK TIGGGDDSFN
51  TFFSETGAGK HVPRAVFDL EPTVIDEVRT GTYRQLFHPE QLITGKEDAA
101 NNYARGHYTI GKEIIDLVLD RIRKLADQCT GLQGFLVFHS FGGGTGSGFT
151 SLLMERLSVD YGKSKLEFS IYPAPQVSTA VVEPYNSILT THTTLEHSDC
201 AFMVDNEAIY DICRRNLDI E RPTYYTNLRL ISQIVSSITA SLRFDGALNV
251 DLTEFQTNLV PYPRIHFPLA TYAPVISA EK AYHEQLSVAE ITNACFEFAN
301 QMVKCDPRHG KYMACLLYR GDVVPKDVNA AIATIKTKRS IQFVDCWPTG
351 PKVGINYQPP TVVPGGDLAK VQRAVCMLSN TTATAEAWAR LDHKFDLMYA
401 KRAFVHWYVG EGMEBGEFSE AREDMAALEK DYEYEVGDSV EGEHEEGEEG
451 YZ

```

↑
↑
↑

424
431
438



GrB cleaves α -tubulin at D438

When we compared the migration pattern of the full length α -tubulin with the migration pattern of grB-cleaved α -tubulin on two-dimensional gels, we noted that the cleaved form of α -tubulin had a more basic isoelectric point (Fig. 3A). Since the C-terminus of α -tubulin contained a cluster of acidic glutamic acid residues, we hypothesized that this region of the protein was removed. As grB cleaves after aspartic acid residues, each of the three most terminal aspartic acid residues (positions 424, 431 or 438) of α -tubulin were mutated to identify the site of cleavage (see Fig. 2A, arrows). Four α -tubulin constructs were generated with N-terminal FLAG epitopes, comprised of either the wild-type α -tubulin sequence or individual mutations of aspartic acid to alanine. These were cloned into the vector pcDNA3.1 hygro and used in transcription/translation reactions. The translation products were treated with grB (1.5 μ g/ml) and the products were resolved by SDS-PAGE and those containing the FLAG epitope were identified on western blots (Fig. 3B).

This analysis demonstrated that α -tubulin could be cleaved by purified grB (Fig. 3B, compare lane 3 with 4), suggesting that grB could either directly cleave α -tubulin or activate another protease present in the reticulocyte lysates that subsequently cleaved α -tubulin. In addition, the α -tubulin

Fig. 2. Amino acid sequence of α -tubulin and western blot analysis of α -tubulin from S100 cell extracts. (A) The amino acid sequence of human α -tubulin (TUBA3). Solid boxes indicated the tryptic peptides that were identified by mass spectrometry analysis. Arrows indicate putative grB cleavage sites. (B) HeLa S100 (lanes 1-6) or Jurkat S100 (lanes 7-12) were treated with the indicated amounts of purified grB (in a 10 μ l reaction volume), in the presence or absence of 100 μ M zVAD-fmk. Protein products were separated by 12% SDS-PAGE and transferred to nitrocellulose. The migration of α -tubulin (upper) and caspase 3 (lower) were assessed by western blot analysis. Full-length α -tubulin is indicated by *, and cleaved α -tubulin by **. GrB-dependent processing of pro-caspase 3 is indicated by p20, and caspase-dependent processing is indicated by p19.

translation product harboring the amino acid substitution of D438 to alanine was no longer cleaved (Fig. 3B, compare lane 4 with 10). Therefore, α -tubulin was cleaved at position D438 directly by grB, or by a grB-activated protease. The cleavage event removed the C-terminal 13 amino acids from α -tubulin. This fragment contains 7 glutamic acid residues (see Fig. 2) and its removal could account for the shift in α -tubulin mobility in both the one-dimensional (Fig. 2B) and two-dimensional (Fig. 3A) SDS-PAGE gels.

α -tubulin is cleaved in cells undergoing cell death

To determine whether α -tubulin was in fact truncated in dying cells, we analyzed HeLa and Jurkat cells that were induced to undergo apoptosis by incubation with CTLs. Treatment of both targets resulted in the production of cleaved α -tubulin (Fig. 4A, compare lane 4 with 5-7 and lane 15 with 16-18). When cells were incubated in the presence of EGTA, which inhibits granular (granzyme B-mediated) killing, no cleaved α -tubulin was detected (Fig. 4A, compare lanes 5-7 with 9-11 and lanes 16-18 with 20-22). This CTL-mediated cleavage of α -tubulin was not dependent on caspase activity, because α -tubulin cleavage was apparent in CTL-treated target cells even in the presence of the caspase inhibitor, zVAD-fmk (Fig. 4B upper, compare lanes 2-4 with 6-8). To ensure that caspase activation was indeed inhibited by incubation with zVAD-fmk, caspase 3 maturation was assessed by western blotting. The caspase-dependent production of the caspase 3 products p19/p17 that were initiated by incubation with CTL, were not produced upon incubation with zVAD-fmk (Fig. 4B lower, compare lanes 2-4 with 6-8), verifying that zVAD-fmk did indeed inhibit caspase activity. As in Fig. 2B, the grB-dependent production of the caspase 3 p20 product was unaffected by the presence of zVAD-fmk, supporting the suggestion that grB could cleave both its substrates pro-caspase 3 and α -tubulin independent of caspase activation.

As the cell-cell killing assay contained both effector and target cells, it was important to determine whether the cleavage of α -tubulin occurred in the CTL, or in the target cell. To that end, target Jurkat cell proteins were metabolically labeled with [35 S]methionine and the cell killing assay was performed as described previously. The α -tubulin was then purified from the cell lysates by immunoprecipitation and the resulting 35 S-labelled proteins were visualized on autoradiograms (Fig. 5A). Immunoprecipitation of Jurkat lysates alone revealed a specific 51 kDa full-length α -tubulin product that was not

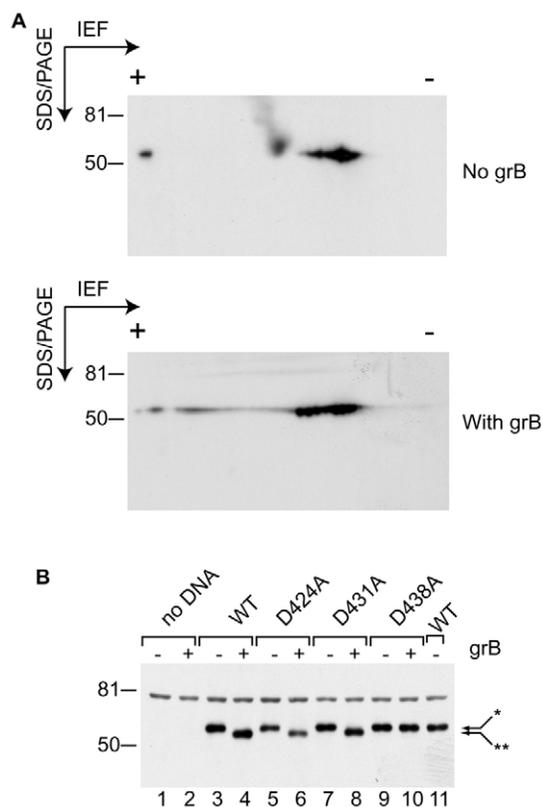


Fig. 3. Two-dimensional western blot of α -tubulin from S100 extracts and western blot analysis of α -tubulin produced from transcription/translation reactions. (A) Jurkat S100 (30 μ g) was incubated with or without purified grB (50 μ M), as indicated. The reactions were then separated on a first dimension IEF IPGphor strip with pH range of 4-6. The second dimension was 12% SDS-PAGE and the presence of α -tubulin was determined by western blotting with anti- α -tubulin. (B) α -tubulin containing FLAG-tagged wild-type amino acid sequence (WT) or point mutants D424A, D431A or D438A, as indicated were created by TNT reactions of the corresponding plasmids. Reactions were incubated with (+) or without (-) purified grB (50 μ M). Protein products were separated on 12% SDS-PAGE for western blot analysis with anti-FLAG.

immunoprecipitated by control antibody (Fig. 5A, compare lanes 1 and 3). In the presence of CTL, however, the α -tubulin was cleaved to the expected molecular weight of 49,000 (Fig. 5A, compare lanes 1 and 2). Immunoprecipitation with a control antibody which would not recognize the endogenous α -tubulin did not result in the recovery of a 51 kDa or 49 kDa α -tubulin product.

Cleaved α -tubulin is not associated with polymerized microtubules

α -tubulin exists in the cell in both a soluble pool of heterodimers with β -tubulin, and also in polymerized microtubules. The determination of which pool is cleaved may reveal clues as to the functional relevance of this cleavage event. To determine whether α -tubulin in the soluble or polymerized pool could be the target of grB cleavage, these two pools of α -tubulin were separated by fractionation into a detergent-soluble (unpolymerized α -tubulin) and a detergent-insoluble phase (polymerized microtubules). These fractions

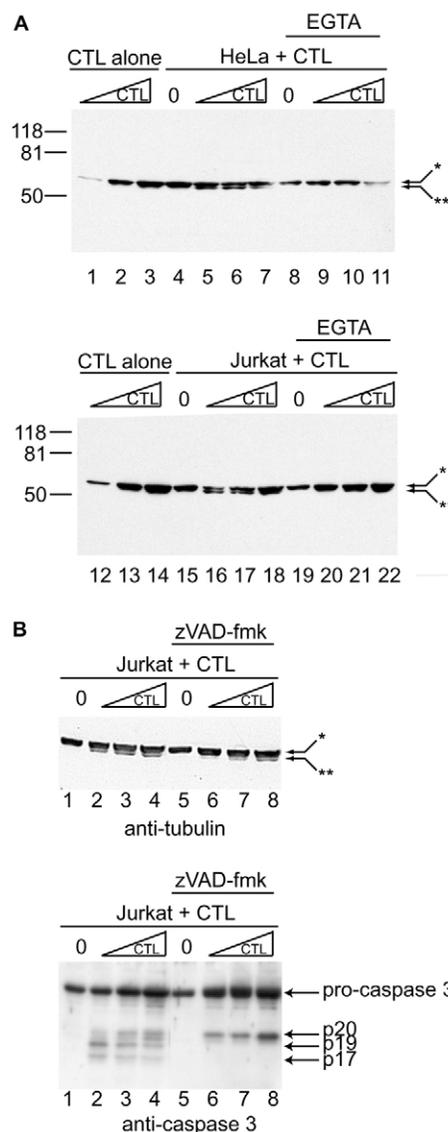


Fig. 4. Western blot analysis of α -tubulin from apoptotic lysates. (A) HeLa cells (lanes 4-11) or Jurkat cells (lanes 15-22) were incubated with CTLs at increasing effector-to-target ratio (E:T) (0.5:1, 1:1 and 2:1) for 4 hours at 37°C. EGTA was included to inhibit granular-mediated killing (lanes 8-11, 19-22). Protein products were separated by 12% SDS-PAGE and analyzed by western blot with anti α -tubulin. Full-length α -tubulin is indicated by *, and cleaved α -tubulin by **. (B) Jurkat cells were incubated with CTLs at increasing E:T (2:1, 5:1 and 10:1) for 4 hours at 37°C. zVAD-fmk (100 μ M final concentration) was included to inhibit caspase activation (lanes 5-8). Protein products were separated by 10% SDS-PAGE and analyzed by western blot with anti α -tubulin (upper panel) and anti-caspase 3 (lower panel). GrB-dependent processing of pro-caspase 3 is indicated by p20, and caspase-dependent processing is indicated by p19 and p17.

were treated with increasing amounts of grB, and α -tubulin cleavage was assessed by western blotting. Both pools of polymerized and soluble α -tubulin could be cleaved by grB (Fig. 5B, compare polymerized fractions in lanes 1-3 and soluble fractions in lanes 4-6). To determine which pools of α -tubulin were cleaved in cells that had been induced to undergo

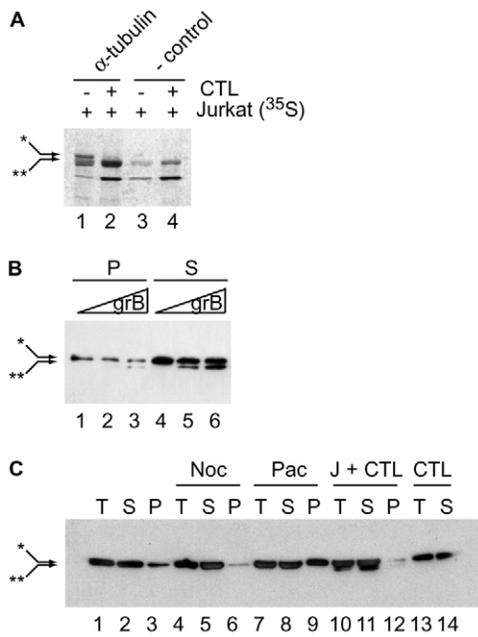


Fig. 5. Immunoprecipitation of α -tubulin from apoptotic lysates. (A) Jurkat cells pre-labelled with [35 S]methionine were incubated with unlabelled effector cells at an E:T of 1:1. Specific full-length α -tubulin is indicated by * in lane 1, and cleaved product by ** in lane 2. Non-specific products are shown in lanes 3 and 4. (B) Jurkat cell lysates were separated into fractions containing soluble α -tubulin (S) and fractions containing polymerized microtubules (P) (see Materials and Methods for details). GrB was added to 5 μ M (lanes 2 and 5) and 10 μ M (lanes 3 and 6). Protein products were separated by 10% SDS-PAGE and analyzed by western blot with anti α -tubulin. (C) Jurkat and CTL (J + CTL) were incubated at an E:T of 1:1 prior to solubilization. The total protein solution (T) was separated into fractions containing soluble α -tubulin (S) and fractions containing polymerized microtubules (P) (lanes 10-12). The efficiency of fractionation to the soluble phase was verified by incubation of Jurkat cells with the microtubule depolymerizing agent nocodazole (Noc, lanes 4-6). The efficiency of fractionation to the polymerized phase was verified by incubation of Jurkat cells with the microtubule polymerizing agent paclitaxel (Pac, lanes 7-9). Protein products were separated on a 12% SDS-PAGE and analyzed by western blot with anti α -tubulin.

cell-mediated apoptosis, cells that had been treated with CTL were fractionated into detergent-soluble and detergent-insoluble phases. Again, the α -tubulin that partitioned into the detergent-insoluble phase constituted the α -tubulin that was present in polymerized microtubules. This was confirmed by the accumulation of α -tubulin in this phase, in the presence of the microtubule-stabilizing drug, paclitaxel (Fig. 5C, 'P'; compare lanes 3 and 9). By contrast, the α -tubulin that partitioned into the detergent-soluble phase constituted the pool of non-polymerized α -tubulin and was confirmed by treatment of the cells with the microtubule destabilizing agent, nocodazole (Fig. 5C, 'S') (Nguyen et al., 1999). When Jurkat cells were incubated with CTL, the appearance of cleaved α -tubulin appeared in the soluble phase (Fig. 5C, compare lanes 11 and 12). Thus, grB-mediated cleavage of α -tubulin caused a loss of full-length α -tubulin from the polymerized microtubule fraction (Fig. 5C, compare lanes 3 and 12) and the appearance of the cleaved form in the soluble fraction (Fig. 5C,

compare lanes 2 and 11). Additional evidence that cleaved α -tubulin was present in the non-polymerized state can be inferred from the accumulation of cleaved α -tubulin in the immunoprecipitation experiments, as this treatment of the cells would favour the recovery of soluble proteins (Fig. 5A, lane 2).

Sequence alignment of α -tubulin isoforms

To assess the overall conservation of the grB cleavage site, α -tubulin isoforms were aligned using sequence data extracted from the ENSEMBL genome browser (NCBI Build 35). In this regard, although the ENSEMBL contained 17 annotations for α -tubulin in the human genome, only 11 appeared authentic. This included previously characterized α -tubulins, exemplified by TUBA2, as well as several potentially novel isoforms (TUBAL3, TUBA5, ENSP00000292748; Fig. 6). For these putative isoforms, multiple transcripts were identified in dbEST that supported the gene prediction, and the genes themselves were characterized by conventional intron/exon structures and contain canonical translation initiation and termination codons. Their overall identity with TUBA2 ranged from 68-94%. This analysis indicates that eight of the α -tubulin proteins have the capacity to undergo grB-mediated cleavage, based on the conservation of D438. Significantly, in the three cases where the cleavage site was not conserved, the proteins were truncated at their C-termini. Notably, the TUBAL3 isoform ended at the first amino acid following the grB cleavage site and could conceivably mimic the proteolyzed form.

Discussion

We have identified that the microtubule protein, α -tubulin, is cleaved by the cytolytic serine protease, grB. This result confirms the observation made by Bredemeyer et al. (Bredemeyer et al., 2004) wherein grB-mediated cleavage of α -tubulin was revealed through a novel proteomic screen. Importantly, we have extended those studies to show that not only is grB capable of cleaving α -tubulin in vitro, but also that α -tubulin is cleaved in cells that are induced to undergo cell death upon treatment with CTL. By mapping the grB cleavage site, we show that grB mediates the removal of a 13-residue fragment at the extreme C-terminus of the protein. Our studies highlight the significance of the C-terminal region of α -tubulin, the implications of which for CTL-mediated cell death are discussed below.

Insights into the function of the α -tubulin C-terminal region may be obtained from analysis of the various tubulin isoforms. Sequence alignment of the available annotated and putative human α -tubulin isoforms indicates they differ mainly at the C-terminal region (Fig. 6) (for review, see Luduena, 1993). Moreover, examination of tubulin isoforms in other model systems has revealed different developmental and tissue-specific expression patterns, suggesting that the variable C-terminus imparts unique activities (Hecht et al., 1988; Pratt and Cleveland, 1988; Villasante et al., 1986). We have also noted an isoform of human α -tubulin with a truncated C-terminus that appears very similar to the grB-cleaved form of tubulin (see Fig. 6; TUBAL3). The characterization of this unique α -tubulin isoform could therefore shed some light onto the functional consequences of grB-cleaved α -tubulin during apoptosis. It will be interesting to see if further studies on this

very intriguing, given that apoptotic cells exhibit massive cytoskeletal changes as they shrink and bleb. This strongly suggests that in addition to the well-documented activation of the mitochondrial apoptotic pathway, other independent, or cooperative, pathways are utilized by grB that ultimately result in the death of the cell. It also suggests that grB is involved not only in the initiation of target cell death by activating pro-apoptotic proteins (procaspase 3 and Bid), but also plays a role in the execution phase of apoptosis by modifying key structural proteins thus enabling the cell to be properly dismantled and eliminated by phagocytosis. Such multiple mechanisms may be critically important to ensure destruction of virus-infected cells that express anti-apoptotic proteins.

Materials and Methods

Cell lines

The human HeLa cell line was maintained in DMEM medium (Gibco BRL Life Technologies) supplemented with 10% fetal calf serum (FCS) (Hyclone), 25 mM HEPES and 100 µg/ml of both penicillin and streptomycin. The human T cell lymphoma Jurkat cell line was maintained in RPMI 1640 medium (RHF) supplemented with 10% FCS, 25 mM HEPES, 100 µM β-mercaptoethanol and 100 µg/ml of both penicillin and streptomycin. Human CTL (hCTL) were generated as previously described (Atkinson et al., 1998) and maintained in RHF containing 80 U/ml of interleukin 2.

Reagents

Human granzyme B was purified from the human Natural Killer cell line, YT-Indy, as previously described (Caputo et al., 1999). The anti-α-tubulin antibodies were from Sigma (clone 512, T 5168) and Cedarlane (DM1A). The anti-FLAG antibody was from Sigma (M5, F4042). The anti-caspase-3 antibody was from Stressgen. Caspase inhibitor zVAD-fmk was purchased from Kamiya and used at a concentration of 100 µM. Nocodazole and paclitaxel were from Sigma and used for 4 hours at a concentration of 10 µM and 2 µM, respectively.

Generation of S100 extracts

Cytosolic S100 extract was generated by lysis of cells, followed by a series of differential centrifugation steps. HeLa cells resuspended in Buffer A sucrose (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose) at a concentration of approximately 4 × 10⁸ cells/ml were lysed by three strokes of a Dounce homogenizer fitted with a B pestle. Nuclei and unbroken cells were removed from the preparation by centrifugation at 1000 g for 10 minutes. Mitochondria were removed from the postnuclear supernatant by centrifugation at 10,000 g for 30 minutes. The postmitochondrial supernatant was then subjected to centrifugation at 100,000 g for 60 minutes to yield the S100 supernatant. Jurkat S100 extracts were obtained in a similar manner, although the Jurkat cells were lysed in Buffer A (100 mM sucrose) containing 0.02% digitonin. All subsequent centrifugation steps were identical to the preparation of HeLa S100.

Mutagenesis and expression constructs

The entire open reading frame of α-tubulin was cloned from the commercially available plasmid pEYFP-tubulin (Clontech) using complementary PCR adaptor primers spanning the initiation and stop codons. N-terminal FLAG sequence was engineered into the 5' primer. Point mutations of the aspartic acid residues at positions D424, D431 and D438 were achieved by use of the QuikChange Kit (Stratagene). All constructs were cloned into the *KpnI/ApaI* site of pcDNA3.1/Hygro (Invitrogen). In vitro transcription/translation reactions were done using a TNT kit (Promega), according to the manufacturer's instructions.

Protein analysis by SDS-PAGE and western blot

For one-dimensional (1-D) gel analysis and immunoblotting, cells were lysed directly into SDS-PAGE gel loading buffer and boiled for 10 minutes. The cell lysates from 1 × 10⁵ cells/lane were subjected to SDS-PAGE analysis. Proteins were transferred to nitrocellulose (Micron Separations) by use of a semi-dry transfer apparatus (Tyler) for 1 hour at 150 mA. Membranes were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and 5% skimmed milk (PBS-T-milk) for 1 hour. Tubulin was detected using a monoclonal anti-α-tubulin antibody (Sigma) at a dilution of 1:2000. FLAG-tagged proteins were detected using anti-FLAG (Sigma, M5) at a dilution of 1:350. Membranes were washed in PBS-T three times and then incubated for 1 hour with a horseradish peroxidase-conjugated secondary anti-mouse antibody at 1:3000 (Biorad) in PBS-T-milk, followed by five washes in PBS-T. Positive signals were visualized using an enhanced chemiluminescence detection system (Amersham).

For two-dimensional IEF/SDS PAGE (2-D) gel analysis, the IPGphor IEF system

(Amersham Pharmacia Biotech) was used. Thirty µg of cell lysate was resuspended in rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer with pH range 4-6, trace of Bromophenol Blue) for 30 minutes prior to transfer into 7 cm ceramic electrode boats. The IPG strip (pH 4-6) was lowered onto the solution and then overlaid with IPG cover fluid. The IPG strips were allowed to rehydrate at room temperature for 15 hours prior to isoelectric focusing at 100 V for 1.5 hours, 500 V for 45 minutes, 1000 V for 45 minutes, 3000 V for 1 hour and 5000 V for 2 hours. The IPG strips were then prepared for the second dimension SDS-PAGE. The strips were equilibrated in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml DTT, trace Bromophenol Blue) for 15 minutes at room temperature. The IPG strip was placed onto the top of a 12% SDS-PAGE that contained no wells or stack and sealed into place using agarose sealing solution (0.5% agarose in SDS electrophoresis buffer with a trace of Bromophenol Blue). Electrophoresis in the second dimension was as described above, for the 1-D gel analysis.

Immunoprecipitation

Jurkat cells were incubated with 0.1 mCi/ml [³⁵S]methionine (Perkin Elmer) overnight in medium and then washed twice in PBS. Jurkat and CTL were incubated together for 4 hours at 37°C at an E:T of 1:1 and a concentration of 8 × 10⁵ each cell type/ml in the presence of concanavalin A (2 µg/ml). The cell pellet was lysed by a 10-minute incubation in lysis buffer (20 mM Tris-HCl pH 8, 1 mM EDTA, 200 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM sodium phosphate, pH 7.4, protease inhibitor cocktail). Lysates were subjected to centrifugation at 14,000 rpm in a microfuge centrifuge for 10 minutes and the supernatant was pre-cleared by incubation with 15 µl each of a 50% slurry of protein A and protein G Sepharose beads for 1 hour at room temperature. Sepharose was removed by a 15-second centrifugation at 1000 rpm in a microfuge. Anti-α-tubulin antibody (DM1A) at 1:20 or control anti-FLAG antibody at 1:20 were added to the supernatant and incubated for at least 16 hours at 4°C. The antibody-protein complexes were recovered from the lysates by binding to Protein A and Protein G Sepharose beads (1 in 4). The beads were washed three times in lysis buffer and then boiled in SDS lysis buffer prior to resolving proteins by 12% SDS-PAGE. The protein gel was transferred to nitrocellulose and labeled proteins were detected by autoradiography using Kodak Biomax MR X-ray film.

α-tubulin fractionation

Jurkat cells that had been incubated in the presence or absence of CTL were resuspended in MSB buffer (85 mM PIPES, pH 6.93, 1 mM EGTA, 1 mM MgCl₂, 2 M glycerol, 0.5% Triton X-100) and incubated for 2 minutes at 37°C. The cells were subjected to a 2-minute centrifugation at 2500 rpm in a microfuge and then separated into a pellet (P) and soluble (S) fractions. Control aliquots of cells were pre-incubated for 4 hours at 37°C with either 10 µM nocodazole or 2 µM paclitaxel, according to Nguyen et al. (Nguyen et al., 1999).

Protein analysis by mass spectrometry

Glassware and plasticware were rinsed with glacial acetic acid and HPLC-grade water before use. HeLa S100 (112 µg) that had been treated with or without 15 µg/ml grB for 30 minutes at 37°C were subjected to SDS-PAGE on a 14% gel and stained with Coomassie Blue. The 51 kDa protein band from the untreated samples and the 49 kDa protein band from the grB-treated samples were excised, minced and submitted for trypsin cleavage and mass spectrometry analysis (MS/MS Q-ToF; Institute for Biomolecular Design, University of Alberta). The resulting peptides were analyzed with the Matrix Science Mascot server (www.matrixscience.com).

We thank Irene Shostak for expert assistance with tissue culture, Lorne Burke for mass spectrometry, Brenda Duggan, Aja Rieger and Russell Quapp for technical support and Simonetta Sipione and Darren Roberts for helpful discussions. This work was supported by grants from the Canadian Institute of Health Research (CIHR), the National Cancer Institute of Canada, and the Howard Hughes Medical Institute awarded to R.C.B. I.S.G. gratefully acknowledges past support from the Alberta Heritage Foundation for Medical Research (AHFMR). D.A.U. is supported by a scholarship from the AHFMR. R.C.B. is a CIHR Distinguished Scientist, a Medical Scientist of the AHFMR, a Canada Research Chair and a Howard Hughes International Scholar.

References

- Adrain, C., Murphy, B. M. and Martin, S. J. (2005). Molecular ordering of the caspase activation cascade initiated by the cytotoxic T lymphocyte/natural killer (CTL/NK) protease granzyme B. *J. Biol. Chem.* **280**, 4663-4673.
- Atkinson, E. A., Barry, M., Darmon, A. J., Shostak, I., Turner, P. C., Moyer, R. W. and Bleackley, R. C. (1998). Cytotoxic T lymphocyte-assisted suicide. Caspase 3 activation is primarily the result of the direct action of granzyme B. *J. Biol. Chem.* **273**, 21261-21266.

- Barry, M. and Bleackley, R. C. (2002). Cytotoxic T lymphocytes: all roads lead to death. *Nat. Rev. Immunol.* **2**, 401-409.
- Barry, M., Heibin, J. A., Pinkoski, M. J., Lee, S. F., Moyer, R. W., Green, D. R. and Bleackley, R. C. (2000). Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol. Cell. Biol.* **20**, 3781-3794.
- Bobiniec, Y., Moudjou, M., Fouquet, J. P., Desbruyeres, E., Edde, B. and Bornens, M. (1998). Glutamylation of centriole and cytoplasmic tubulin in proliferating non-neuronal cells. *Cell. Motil. Cytoskeleton* **39**, 223-232.
- Bredemeyer, A. J., Lewis, R. M., Malone, J. P., Davis, A. E., Gross, J., Townsend, R. R. and Ley, T. J. (2004). A proteomic approach for the discovery of protease substrates. *Proc. Natl. Acad. Sci. USA* **101**, 11785-11790.
- Browne, K. A., Johnstone, R. W., Jans, D. A. and Trapani, J. A. (2000). Filamin (280-kDa actin-binding protein) is a caspase substrate and is also cleaved directly by the cytotoxic T lymphocyte protease granzyme B during apoptosis. *J. Biol. Chem.* **275**, 39262-39266.
- Caputo, A., Parrish, J. C., James, M. N., Powers, J. C. and Bleackley, R. C. (1999). Electrostatic reversal of serine proteinase substrate specificity. *Proteins* **35**, 415-424.
- Cross, D., Dominguez, J., Maccioni, R. B. and Avila, J. (1991). MAP-1 and MAP-2 binding sites at the C-terminus of beta-tubulin. Studies with synthetic tubulin peptides. *Biochemistry* **30**, 4362-4366.
- Darmon, A. J., Nicholson, D. W. and Bleackley, R. C. (1995). Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* **377**, 446-448.
- Duan, J. and Gorovsky, M. A. (2002). Both carboxy-terminal tails of alpha- and beta-tubulin are essential, but either one will suffice. *Curr. Biol.* **12**, 313-316.
- Goping, I. S., Barry, M., Liston, P., Sawchuk, T., Constantinescu, G., Michalak, K. M., Shostak, I., Roberts, D. L., Hunter, A. M., Korneluk, R. et al. (2003). Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. *Immunity* **18**, 355-365.
- Hecht, N. B., Distel, R. J., Yelick, P. C., Tanhauser, S. M., Driscoll, C. E., Goldberg, E. and Tung, K. S. (1988). Localization of a highly divergent mammalian testicular alpha tubulin that is not detectable in brain. *Mol. Cell. Biol.* **8**, 996-1000.
- Heibin, J. A., Goping, I. S., Barry, M., Pinkoski, M. J., Shore, G. C., Green, D. R. and Bleackley, R. C. (2000). Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. *J. Exp. Med.* **192**, 1391-1402.
- Henkart, P. A. (1985). Mechanism of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* **3**, 31-58.
- Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K. J., Podack, E. R., Zinkernagel, R. M. and Hengartner, H. (1994). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369**, 31-37.
- Kojima, H., Shinohara, N., Hanaoka, S., Someya-Shirota, Y., Takagaki, Y., Ohno, H., Saito, T., Katayama, T., Yagita, H., Okumura, K. et al. (1994). Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity* **1**, 357-364.
- Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski, D. J. et al. (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278**, 294-298.
- Larcher, J. C., Boucher, D., Lazereg, S., Gros, F. and Denoulet, P. (1996). Interaction of kinesin motor domains with alpha- and beta-tubulin subunits at a tau-independent binding site. Regulation by polyglutamylation. *J. Biol. Chem.* **271**, 22117-22124.
- Li, L. Y., Luo, X. and Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412**, 95-99.
- Lieberman, J. (2003). The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat. Rev. Immunol.* **3**, 361-370.
- Littauer, U. Z., Giveon, D., Thierauf, M., Ginzburg, I. and Ponstingl, H. (1986). Common and distinct tubulin binding sites for microtubule-associated proteins. *Proc. Natl. Acad. Sci. USA* **83**, 7162-7166.
- Lord, S. J., Rajotte, R. V., Korbutt, G. S. and Bleackley, R. C. (2003). Granzyme B: a natural born killer. *Immunol. Rev.* **193**, 31-38.
- Lowin, B., Beermann, F., Schmidt, A. and Tschopp, J. (1994). A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA* **91**, 11571-11575.
- Ludena, R. F. (1993). Are tubulin isotypes functionally significant. *Mol. Biol. Cell* **4**, 445-457.
- Martin, S., Amarante-Mendes, G., Shi, L., Chuang, T., Casiano, C., O'Brien, G., Fitzgerald, P., Tan, E., Bokoch, G., Greenberg, A. et al. (1996). The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. *EMBO J.* **15**, 2407-2416.
- Mitchison, T. and Kirschner, M. (1984). Dynamic instability of microtubule growth. *Nature* **312**, 237-242.
- Mollinedo, F. and Gajate, C. (2003). Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis* **8**, 413-450.
- Nagata, S. and Golstein, P. (1995). The Fas death factor. *Science* **267**, 1449-1456.
- Nguyen, H., Gruber, D. and Bulinski, J. (1999). Microtubule-associated protein 4 (MAP4) regulates assembly, protomer-polymer partitioning and synthesis of tubulin in cultured cells. *J. Cell Sci.* **112**, 1813-1824.
- Nogales, E., Wolf, S. G. and Downing, K. H. (1998). Structure of the alpha beta tubulin dimer by electron crystallography. *Nature* **391**, 199-203.
- Pratt, L. F. and Cleveland, D. W. (1988). A survey of the alpha-tubulin gene family in chicken: unexpected sequence heterogeneity in the polypeptides encoded by five expressed genes. *EMBO J.* **7**, 931-940.
- Roberts, D. L., Goping, I. S. and Bleackley, R. C. (2003). Mitochondria at the heart of the cytotoxic attack. *Biochem. Biophys. Res. Commun.* **304**, 513-518.
- Russell, J. H. and Ley, T. J. (2002). Lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* **20**, 323-370.
- Sharif-Askari, E., Alam, A., Rheume, E., Beresford, P. J., Scotto, C., Sharma, K., Lee, D., DeWolf, W. E., Nuttall, M. E., Lieberman, J. et al. (2001). Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. *EMBO J.* **20**, 3101-3113.
- Shi, L., Kraut, R. P., Aebersold, R. and Greenberg, A. H. (1992). A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J. Exp. Med.* **175**, 553-566.
- Stinchcombe, J. C., Bossi, G., Booth, S. and Griffiths, G. M. (2001). The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* **15**, 751-761.
- Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M. et al. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441-446.
- Sutton, V. R., Davis, J. E., Cancilla, M., Johnstone, R. W., Ruefli, A. A., Sedelies, K., Browne, K. A. and Trapani, J. A. (2000). Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J. Exp. Med.* **192**, 1403-1414.
- Sutton, V. R., Wowk, M. E., Cancilla, M. and Trapani, J. A. (2003). Caspase activation by granzyme B is indirect, and caspase autoprocessing requires the release of proapoptotic mitochondrial factors. *Immunity* **18**, 319-329.
- Thomas, D. A., Du, C., Xu, M., Wang, X. and Ley, T. J. (2000). DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. *Immunity* **12**, 621-632.
- Tian, G., Bhamidipati, A., Cowan, N. J. and Lewis, S. A. (1999). Tubulin folding cofactors as GTPase-activating proteins. GTP hydrolysis and the assembly of the alpha/beta-tubulin heterodimer. *J. Biol. Chem.* **274**, 24054-24058.
- Trapani, J. A. and Sutton, V. R. (2003). Granzyme B: pro-apoptotic, antiviral and antitumor functions. *Curr. Opin. Immunol.* **15**, 533-543.
- Trapani, J. A., Jans, D. A., Jans, P. J., Smyth, M. J., Browne, K. A. and Sutton, V. R. (1998). Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. *J. Biol. Chem.* **273**, 27934-27938.
- Van de Craen, M., Van den Brande, I., Declercq, W., Irmeler, M., Beyaert, R., Tschopp, J., Fiers, W. and Vandenebeele, P. (1997). Cleavage of caspase family members by granzyme B: a comparative study in vitro. *Eur. J. Immunol.* **27**, 1296-1299.
- Villasante, A., Wang, D., Dobner, P., Dolph, P., Lewis, S. A. and Cowan, N. J. (1986). Six mouse alpha-tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. *Mol. Cell. Biol.* **6**, 2409-2419.
- Waterhouse, N. J., Clarke, C. J., Sedelies, K. A., Teng, M. W. and Trapani, J. A. (2004). Cytotoxic lymphocytes: instigators of dramatic target cell death. *Biochem. Pharmacol.* **68**, 1033-1040.
- Wolf, B. B., Schuler, M., Echeverri, F. and Green, D. R. (1999). Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. *J. Biol. Chem.* **274**, 30651-30656.
- Xia, L., Hai, B., Gao, Y., Burnette, D., Thazhath, R., Duan, J., Bre, M. H., Levilliers, N., Gorovsky, M. A. and Gaertig, J. (2000). Polyglycylation of tubulin is essential and affects cell motility and division in *Tetrahymena thermophila*. *J. Cell Biol.* **149**, 1097-1106.
- Yannelli, J. R., Sullivan, J. A., Mandell, G. L. and Engelhard, V. H. (1986). Reorientation and fusion of cytotoxic T lymphocyte granules after interaction with target cells as determined by high resolution cinemicrography. *J. Immunol.* **136**, 377-382.