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Identification of the BCL2/Adenovirus E1B-19K protein-interacting protein 2 (BNIP-2) as a granzyme B target during human natural killer cell-mediated killing.

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SYNOPSIS

Cytotoxic lymphocytes eliminate infected cells and tumours via the perforin mediated delivery of pro-apoptotic serine proteases known as granzymes. Granzyme B triggers apoptosis *via* the cleavage of a repertoire of cellular proteins, leading to caspase activation and mitochondrial depolarisation. A simple bioinformatics strategy identified a candidate granzyme B cleavage site in the widely expressed BCL2/Adenovirus E1B 19K protein-interacting protein-2 (BNIP-2). Granzyme B cleaved recombinant BNIP-2 *in vitro* and endogenous BNIP-2 was cleaved during the NK cell mediated killing of tumour cells. Cleavage required the site identified in the bioinformatics screen and was caspase-independent. Expression of either full length BNIP-2 or a truncated molecule mimicking the granzyme B cleaved form was pro-apoptotic and led to the caspase-dependent cleavage of BNIP-2 at a site distinct from granzyme B cleavage. Inhibition of BNIP-2 expression did not affect the susceptibility to NK cell-mediated killing. Furthermore, target cells in which BID expression was inhibited also remained highly susceptible to NK cell-mediated killing, revealing redundancy in the pro-apoptotic response to human cytotoxic lymphocytes. Such redundancy reduces the opportunity for escape from apoptosis induction and maximises the chances of immune-mediated clearance of infected cells or tumour cells.

Key words: Natural Killer (NK) cell, cytotoxic lymphocyte, granzyme, caspase, apoptosis, BID.

Abbreviations: BNIP-2, BCL2/Adenovirus E1B-19K protein-interacting protein 2; BID, BH3-interacting domain death agonist; CTL, cytotoxic T lymphocyte; NK cell, natural killer cell; poly(ADP ribose) polymerase-1, PARP-1; small hairpin RNA, shRNA; small interfering RNA, siRNA; FACS, Fluorescence activated cell sorting; E:T ratio, effector:target ratio; IL-2, interleukin-2; glutathione-S-transferase, GST; Cell Tracker Green, CTG; Cell Tracker Orange, CTO.

INTRODUCTION

Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) kill infected cells and tumour cells by induction of the apoptotic pathway. This occurs either by the ligation of target cell death receptors (such as Fas or TRAIL-receptor) or by the perforin-mediated delivery of granzymes, a family of pro-apoptotic serine proteases [1, 2]. The proteolytic activity of granzymes allows them to cleave an array of target cell proteins that results in the induction of the apoptotic pathway and the dismantling of cell structure and function [3]. Granzyme B has particularly potent pro-apoptotic activity and, unique amongst the granzymes, cleaves on the C-terminal side of aspartate residues present within a tetrapeptide recognition motif [1]. Granzyme B induces apoptosis by cleavage of proteins including pro-caspases-3 and -7 and the BH3-interacting domain death agonist BID [2-7]; BID cleavage generates a truncated form, tBID, which translocates to the mitochondria and mediates the release of cytochrome C and the formation of the caspase-activating apoptosome [2]. Many granzyme B substrates have been identified using a variety of biochemical approaches, including studies of endogenous proteins as well as substrates encoded in cDNA libraries [4, 8-12]. At least one substrate, HSC70 interacting protein (HIP), was identified using a bioinformatics-based screen to detect homology to the polypeptide loop containing the granzyme B cleavage site within BID [13]. Here we have used a sequence search tool to identify proteins containing the tetrapeptide motif IEAD, the site at which granzyme B cleaves BID. We show that BNIP-2 contains this motif and that granzyme B cleaves at this site *in vitro* and in cells undergoing attack by NK cells.

MATERIALS AND METHODS

Identification and expression of BNIP-2

IEAD motif containing proteins were identified using the Mass Spectrometry DataBase (MSDB) search tool (www.proteomics.leeds.ac.uk/bioinf/delphos.html), designed to match short peptide sequences obtained by mass spectrometry to their corresponding proteins. Human BNIP-2 was amplified from an Expressed Sequence Tag clone (Accession Number BC002461) and a c-myc epitope tag added to the C-terminus. Three derivatives were produced, one containing the wild-type IEAD motif, BNIP-2/D²⁸, one in which the aspartate residue of IEAD was mutated to alanine, BNIP-2/A²⁸ (using a QuikChange XL Site-Directed Mutagenesis Kit, Stratagene) and an N-terminally truncated derivative (produced by PCR) that mimicked the granzyme B cleaved product, denoted (g)BNIP-2. The (g)BNIP-2 sequence was engineered such that the first four amino acids from the N-terminus were MAIL, where the isoleucine residue is I²⁹ of BNIP-2, located on the C-terminal side of the predicted granzyme B cleavage site at D²⁸. Mammalian expression constructs were generated using pcDNA3 (Invitrogen). For *E. coli* expression, BNIP-2 derivatives were fused to the C-terminus of glutathione-S-transferase (GST) using pGEX4T3 (GE Healthcare), and proteins harvested three hours post-IPTG induction from cells growing exponentially at 20°C.

Lysate production, cleavage assays and immunoblotting

Bacterial lysates were prepared using the BugBuster system (Novagen) and cleavage reactions performed directly on the glutathione bead-bound material. Mammalian cell

lysates were made using 50mM Tris-HCl, 150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, pH 7.5. For cleavage reactions, GST-BNIP-2myc derivatives were incubated with mammalian cell lysates (equivalent to 10^7 cells/ml) or recombinant human granzyme B (Calbiochem) for 10 minutes at 37°C and reactions stopped by addition of SDS loading buffer. Protease inhibitors were omitted from all *in vitro* cleavage assays. BNIP-2 cleavage was analysed by immunoblotting using an antibody against the endogenous protein [14] or, when testing transfectants, an anti-myc antibody (Cell Signaling Technologies) to detect the C-terminal epitope tag. Other antibodies used included PARP-1 (Cell Signaling Technologies), granzyme B (Pharmingen), BID (Cell Signaling Technologies) and Actin (Sigma).

RNA interference

BNIP-2 expression was inhibited using a commercially available system containing four plasmids encoding different shRNA molecules targeting BNIP-2 (Origene: Catalogue number TR314451). These plasmids were transfected into HeLa cells using Fugene (Roche) and stable transfectants selected using puromycin. Control transfections included a plasmid encoding a control shRNA (a scrambled sequence based on GFP) and an empty vector (Origene; catalogue numbers TR30012 and TR20003 respectively). Clonal BNIP-2 shRNA clones were obtained by limiting dilution of transfectants derived from shRNA-2 of this set (which demonstrated the greatest inhibition when assessed by immunoblotting of the pool). Selected clones had a >75% reduction in BNIP-2 protein expression as assessed by immunoblotting and densitometry. Inhibition of BID expression used a commercially available siRNA (Silencer siRNA from Applied Biosystems; catalogue number 4390824) and a control siRNA (from Eurogentec; ACGUGACACGUUCGGAGAATT), both at a final concentration of 1nM, transiently transfected into HeLa cells using Oligofectamine (Invitrogen). Knockdown was assessed by immunoblotting after 48, 72 and 96 hours and was greater than 80% (as assessed by immunoblotting and densitometry) at all of these time points (data not shown). The 72 hour time point was chosen for killing assays (described below), with the knockdown in the same batch of cells confirmed by immunoblotting.

Cytotoxic lymphocytes and NK cell killing assays

NK cells and CTL were purified as previously described [15] and activated using 50 units/ml interleukin (IL)-2 for 3-5 days. HeLa cells were transiently transfected (using Fugene, Roche) with BNIP-2myc derivatives in the presence of the caspase inhibitor zVAD-fmk at 0.1mM (Calbiochem) and used approximately 24 hours post-transfection. Transfected HeLa cells were mixed with NK cells, or 5mM EGTA treated NK cells [16], at the appropriate Effector:Target (E:T) ratio, and either lysed immediately, co-cultured for 5 hours before lysis and immunoblotting or used in flow cytometric killing assays as previously described [15]. In some experiments the NK cell line NK92MI was used (from the American Type Culture Collection). This cell line is a derivative of the NK cell line NK92 and is stably transfected with an IL-2 cDNA, allowing growth in the absence of exogenous IL-2 [17].

Fluorescence activated cell sorting (FACS)

NK92MI cells were labelled with Cell Tracker Green and Daudi cells with Cell Tracker Orange (both dyes from Molecular Probes/Invitrogen), allowing identification in the FL1 and FL2 channels respectively of a Dako Cytomation MoFlo cell sorter. NK and Daudi cells were mixed at an E:T ratio of 3:1 (approximately 6-9

million NK cells and 2-3 million target cells) and co-cultured for 2.5 or 4 hours, or cultured alone for 4 hours, and then sorted. Sorted populations were re-analysed by FACS for their purity, which was 95-99%. Sorted cells were processed and analysed by immunoblotting as described above. Lysate from approximately 1 million sorted cells was analysed per lane of the immunoblot.

RESULTS

Identification of BNIP-2 as a candidate granzyme B substrate

We used a protein sequence database search tool to identify proteins that contained an IEAD motif, the granzyme B recognition and cleavage motif present in BID. Programmes such as the Basic Local Alignment Search Tool (BLAST) are not suitable for matching very short sequences and instead we employed a novel strategy. Protein sequencing by mass spectrometry generates short peptide sequences that are then matched to corresponding proteins via tools such as the Mass Spectrometry DataBase (MSDB). We searched MSDB for proteins containing the IEAD motif and filtered the output for human proteins. This analysis returned the prototype BID sequence, the known granzyme B substrates topoisomerase I and pro-caspase-10 [4, 18], and other proteins, including the BCL2/Adenovirus E1B 19K protein-interacting protein-2 (BNIP-2, also known as NIP2 and NIP21). BNIP-2 was originally identified as a widely expressed binding partner of the adenovirus anti-apoptotic protein, E1B 19K, as well as its cellular homologue, BCL2 [19]. BNIP-2 has pro-apoptotic activity [20, 21] and is a caspase substrate [22, 23] making it a good candidate for granzyme B cleavage. Analysis of the BNIP-2 sequence revealed the presence of the IEAD motif close to the *N*-terminus of BNIP-2 (Figure 1). Furthermore, use of genome sequence databases to analyse BNIP-2 orthologues in a variety of species revealed that the BNIP-2 molecule is very highly conserved in vertebrate evolution and that the IEAD motif was present in all of the mammalian species analysed, but not conserved in the chicken or puffer fish sequences (Figure 1). The conservation of the IEAD motif, a known granzyme B cleavage site, in the mammalian BNIP-2 protein, coupled with the involvement of BNIP-2 in the apoptotic pathway, suggested that BNIP-2 was a good candidate for further investigation.

A FACS-based approach to analyse protease cleavage during NK cell killing

We first tested cleavage of endogenous BNIP-2 during NK cell mediated killing of target cells. We chose an approach that would allow cleavage to be analysed following delivery of granzymes via the authentic granule exocytosis pathway. One difficulty with such assays is that substrate molecules may be expressed by both the target and effector cells; conventional immunoblotting cannot therefore determine whether any cleaved species originate from the target cell, the effector cell, or both cell-types. We therefore developed a FACS-based approach in which NK cells and target cells were co-cultured, as in a conventional cytotoxicity assay, and then separated by FACS, allowing proteins from these two populations to be analysed separately. The NK cells and target cells were labelled with Cell Tracker Green and Cell Tracker Orange respectively, and co-cultured for 2.5 or 4 hours. The effector and target cell populations were then separated by FACS based on their fluorescent dye content (Figure 2). A small fraction of each sorted population was re-analysed by flow cytometry, revealing that the two cell-types were recovered with 95-99% purity (Figure 2). The remainder of the sorted cells were used to generate whole cell lysates which were then analysed by immunoblotting to test for cleavage of BNIP-2

and PARP-1, a well defined substrate of both granzyme B and caspases [24, 25]. PARP-1 cleavage increased substantially in the target cells during a four-hour co-culture with NK cells. Cleavage of PARP-1 was also evident in NK cells, but this cleavage did not increase during the co-culture and the cleaved PARP-1 species remained in the minority in the NK cells during the co-culture period. Analysis of BNIP-2 revealed two species migrating at approximately 50 kDa in both the target and NK cell populations, consistent with previous data [14, 26]; co-culture resulted in the loss of the slower migrating species in the target cells, whilst it remained intact in the NK cells. These data suggested that target cell BNIP-2 was cleaved during NK cell attack.

Cleavage of recombinant BNIP-2 at the IEAD motif by granzyme B

We next characterised the cleavage of BNIP-2 in more detail. A GST-BNIP-2 fusion protein, with a C-terminal c-myc epitope tag was expressed in *E. coli* and used as a substrate in cleavage assays with lysates derived from cytotoxic lymphocytes and in assays using recombinant granzyme B (Figure 3). Two forms of BNIP-2 were tested for cleavage, a wild-type derivative with the aspartate residue at position 28 (BNIP-2/D²⁸), and a mutant form, in which this residue was altered to an alanine residue (BNIP-2/A²⁸), to prevent cleavage by the aspartase activity of granzyme B. The BNIP-2/D²⁸ was cleaved rapidly (within 10 minutes) by lysates derived from human NK cells which express high levels of granzyme B (Figure 3B and C). This generated a cleaved form (denoted gBNIP-2) of a size consistent with cleavage at the D²⁸ residue. In contrast, no cleavage was observed with BNIP-2/A²⁸ derivative. Lysates derived from CTL and NK cells cleaved BNIP-2/D²⁸ in direct proportion to the amount of granzyme B they contained, whereas lysates derived from the B-cell line Daudi, which does not express granzyme B, failed to cleave the wild-type substrate (Figure 3C). The sequence specificity of this cleavage strongly implicated granzyme B in this reaction. Use of recombinant granzyme B in these assays demonstrated rapid cleavage of BNIP-2/D²⁸ but not BNIP-2/A²⁸ at this site, indicating that BNIP-2 was cleaved by granzyme B at the IEAD motif identified in the sequence search (Figure 3D).

Cleavage of BNIP-2 by caspase-dependent and independent pathways

The FACS-based assay suggested that endogenous BNIP-2 was cleaved during NK cell killing (Figure 2) and granzyme B-mediated cleavage of BNIP-2 at the IEAD motif *in vitro* supports this. We wanted to test whether cleavage during NK cell killing was dependent on the IEAD motif and, since caspases can cleave BNIP-2 [22, 23], it was important to test whether cleavage of BNIP-2 could occur in the absence of granzyme-mediated caspase activation. We expressed the D²⁸ and A²⁸ derivatives of c-myc epitope tagged BNIP-2 in HeLa cells and used these transfected cells as targets in cytotoxicity assays. This simplified the detection of the cleaved species and enabled the cleavage site to be mapped in the context of a killing assay. However, over-expression of BNIP-2 has been previously shown to be pro-apoptotic [20]. In agreement with these findings, transient expression of BNIP-2 in HeLa cells was associated with the caspase-dependent induction of PARP-1 cleavage. Furthermore, BNIP-2 itself was cleaved in a caspase dependent manner, as revealed by the sensitivity of the cleavage reaction to the caspase inhibitor zVAD-fmk (Figure 4A). However, caspase cleavage of BNIP-2 generated a C-terminal fragment approximately 20 kDa smaller than the intact BNIP-2 protein, whereas granzyme B

cleavage occurred much closer to the *N*-terminus, removing approximately 5 kDa of *N*-terminal sequence. Furthermore, both the D²⁸ and A²⁸ constructs were cleaved via caspases, confirming that granzyme B and caspases generated distinct cleavage products.

Overexpression of *C*-terminal deletion mutants of BNIP-2 has revealed that its pro-apoptotic function requires an intact BCH domain [27]. Granzyme B cleaves close to the *N*-terminus and releases a large *C*-terminal fragment including the BCH domain. Expression of an *N*-terminal truncation mutant, (g)BNIP-2, that mimicked the granzyme B cleavage product gBNIP-2, was more difficult to detect by immunoblotting than the D²⁸ derivative, yet accumulated in the transfected cells and retained the ability to induce PARP-1 cleavage. This reveals that the *C*-terminal fragment of BNIP-2 released by granzyme B cleavage retains pro-apoptotic function (Figure 4B).

The ability to inhibit caspase activity allowed us to assay cleavage of BNIP-2 in transfected targets during NK cell attack and to compare the products of granzyme B and caspase-mediated cleavage. HeLa cells transiently transfected with BNIP-2myc, in the presence of zVAD-fmk, were incubated with NK cells and lysates prepared either immediately, or after five hours of co-culture. The BNIP-2/D²⁸ derivative was cleaved during NK cell attack but the BNIP-2/A²⁸ molecule was resistant (Figure 5A). Thus, BNIP-2 was cleaved at the IEAD motif, the site of granzyme B cleavage *in vitro*, in a caspase-independent manner. The absence of BNIP-2 cleavage when NK cells and HeLa cells were lysed immediately after mixing indicated that BNIP-2 cleavage occurred due to the perforin-mediated transfer of granzymes to the target cell, rather than as a result of a post-lysis cleavage event. To confirm this, we repeated the killing assay using NK cells treated with EGTA, which inhibits killing by preventing the calcium-dependent polymerisation of perforin [16]. Under these conditions, BNIP-2/D²⁸ was not cleaved (Figure 5A) and there was a substantial reduction in NK cell mediated killing of the targets as judged by a flow cytometric assay (Figure 5B). Nevertheless, granzyme B remained active in the presence of EGTA (Figure 5C). This NK cell cytotoxicity assay was repeated using the NK cell line NK92 and the resulting BNIP-2 cleavage product was compared to the fragment resulting from caspase-mediated cleavage of BNIP-2. The results confirm that caspase-dependent and caspase-independent cleavage of BNIP-2 occurs at distinct sites, indicating that granzyme B cleaves at the IEAD motif, whilst caspase cleavage occurs towards the *C*-terminus of the molecule (Figure 5D).

Target cell BNIP-2 and BID are dispensable for killing by human NK cells

To analyse whether target cell BNIP-2 was required for NK cell-mediated killing we generated stable clones of HeLa cells in which BNIP-2 expression was inhibited by shRNA interference. Pools of shRNA transfectants exhibited only a modest reduction in BNIP-2 protein expression. However, cloning identified two lines (Clones 2 and 4; Figure 6A) with a 75% reduction of BNIP-2 protein expression. In cytotoxicity assays, clones with reduced BNIP-2 expression did not show significantly altered susceptibility to killing by IL-2 activated NK cells compared to controls (Figure 6B). One possibility was that, despite its cleavage by granzyme B and caspases, BNIP-2 may not actively participate in apoptosis induced by NK cells. Alternatively, redundancy in NK cell-induced apoptotic pathways will mask any phenotype caused by loss of a single molecule. To analyse this potential redundancy we analysed the effect of BID inhibition on susceptibility to NK cells. Like BNIP-2, BID is cleaved by both granzyme B and caspases and plays an important role in granzyme B-induced

killing [5-7]. Transient siRNA-mediated inhibition of BID expression in HeLa cells produced an 85% reduction in BID protein expression (Figure 6C). However, when used as targets in an NK cell cytotoxicity assay, these BID-deficient HeLa cells showed no alteration in their susceptibility to NK cell mediated killing (Figure 6D). Thus, neither target cell BID nor BNIP-2 were required for killing by NK cells.

DISCUSSION

We have identified the BNIP-2 molecule as a granzyme B substrate. The BNIP-2 molecule has previously been identified as a caspase substrate raising the possibility that BNIP-2 cleavage could be secondary to granzyme-mediated caspase activation [22, 23]. However, recombinant granzyme B cleaved BNIP-2 expressed in *E. coli* (Figure 3D) and cleavage of BNIP-2 during NK cell-mediated killing occurred in the presence of caspase-inhibition (Figure 5). Cleavage by granzyme B *in vitro*, or during NK cell-mediated killing, required the D²⁸ residue (Figures 3D and 5), whereas cleavage associated with BNIP-2 over-expression was D²⁸ independent and generated a smaller cleavage product (Figure 5), consistent with caspase cleavage occurring on the C-terminal side of the IEAD motif [23]. These results all support cleavage of BNIP-2 by granzyme B at the IEAD motif identified in our database search. Furthermore, in a large scale proteomics-based screen, Van Damme *et al* [28] identified novel N-termini exposed as a result of digestion with recombinant granzyme B; one of the species identified was derived from BNIP-2, with an N-terminus located at residue I²⁹, consistent with cleavage at the D²⁸ residue identified as the granzyme B cleavage site here. Furthermore, cleavage of BNIP-2 occurred using recombinant granzyme B of both mouse and human origin [28]. This large-scale screen identified some three hundred or so human proteins that were cleaved in lysates by purified granzyme B. Far fewer substrates were detected when the same techniques were applied following NK cell-mediated killing and BNIP-2 was not amongst the cleaved molecules detected. However, several well-characterised granzyme B substrates were also absent (for example, no caspase products were detected), possibly due to the subsequent degradation of cleaved substrates by other proteases. Our FACS-based approach revealed that endogenous target cell BNIP-2 was degraded during NK cell-mediated killing, whereas BNIP-2 in the NK cell was unaffected (Figure 2). It is interesting that only one species of BNIP-2 was targeted by NK cell killing (Figure 2). Multiple BNIP-2 protein species may relate either to post-translational modifications [14], or to the presence of alternatively spliced BNIP-2 mRNA molecules.

The BNIP-2 molecule was originally identified as a binding partner of the adenovirus anti-apoptotic protein E1B-19K and its cellular functional homologue, BCL2 [19]. The expression of BNIP-2myc induced caspase-dependent PARP-1 cleavage (Figure 4), in agreement with previous data showing that BNIP-2 over-expression was pro-apoptotic [20, 21]. It is tempting to speculate that the pro-apoptotic effects of BNIP-2 may normally be counter-balanced by binding to the anti-apoptotic BCL2 molecule, with BNIP-2 over-expression tipping the balance in favour of apoptosis; co-transfection of BNIP-2 and BCL2 supports this model [21]. Pro-apoptotic activity of BNIP-2 requires an intact BCH domain [27]. Caspase cleavage of BNIP-2 occurs between the IEAD motif and the BCH domain and Valencia *et al.* have suggested a model in which caspase cleavage releases a pro-apoptotic fragment [23]. We show that expression of the surrogate fragment of granzyme B cleavage, (g)BNIP-2, was pro-apoptotic (Figure 4B), revealing that granzyme B cleavage does

not inhibit the pro-apoptotic activity and consistent with the release of the BCH domain. Expression of (g)BNIP-2 was weak compared to the full length molecule but both induced a similar level of PARP cleavage. This suggests that, on a per-molecule basis, the pro-apoptotic activity of BNIP-2 might be enhanced by granzyme B cleavage; this is currently under test. Why cleave BNIP-2 with granzyme B directly when granzyme B will activate the caspase pathway, allowing activated caspases to cleave BNIP-2? Whilst the answer to this question is unclear, such a mechanism does not set a precedent; the proapoptotic protein BID, which shares the IEAD motif, is cleaved by both granzyme B and caspases, as are other substrates such as PARP-1, MCL1, Filamin and ICAD [3, 25, 29-31]. Indeed, PARP-1 is also cleaved by granzyme A [32].

Target cell expression of BNIP-2 or BID was dispensable for NK cell-mediated killing of HeLa cells (Figure 6C and D). In the case of BID, this reflects redundancy in the pro-apoptotic pathways; such redundancy is not confined to apoptosis induction by cytotoxic lymphocytes, since BID is dispensable for apoptosis induced by a variety of stimuli [33]. Over-expression of BCL2 in human cells confers resistance to granzyme B (as does deletion of BID in mouse cells, [7]), but this does not render target cells resistant to attack by cytotoxic lymphocytes [34]. Moreover, BID deficient mouse cells are killed by mouse CTL expressing granzyme B [35]. Similarly, our results show that human BID is not required for killing by NK cells. Multiple granzymes are expressed by IL-2 activated human NK cells [36] and all human granzymes participate in the induction of cell death via various routes [1-3, 37]. Furthermore, granzyme B-deficient mouse cytotoxic cells retain pro-apoptotic activity [38]. Multiple granzymes and redundancy in the pro-apoptotic pathways targeted by cytotoxic lymphocytes may have evolved to combat pathogen-encoded evasion strategies. The expression of BNIP-2 is inhibited by Cocksackievirus infection [20]. Furthermore, adenovirus infection (and E1A expression) is pro-apoptotic, but adenovirus inhibits apoptosis via E1B-19K mediated sequestration of BAX and BAK [39, 40]. Similarly, binding of BNIP-2 by E1B-19K may limit BNIP-2 cleavage and subsequent apoptotic events. Extensive redundancy makes it difficult to assess the functional contribution of individual components (as discussed in [41]). Hence, determining whether BNIP-2 (or many other identified substrates) participate directly in apoptosis induction or aid the dismantling of cellular structure and function is challenging. For example, BNIP-2 also interacts (via its BCH domain) with the Rho family GTPase CDC42 and the CDC42 GTPase activating-protein CDC42GAP, also known as p50-RhoGAP [14, 42, 43]. CDC42 is best known for its role in regulating cell motility and polarity via cytoskeletal remodelling [44] and BNIP-2 over-expression has previously been associated with CDC42 dependent changes in cell morphology [45]. It is possible that BNIP-2 cleavage by granzyme B (or caspases) may give rise to cytoskeletal changes. Interestingly, both CDC42 and CDC42GAP have been implicated in the regulation of apoptosis, including the susceptibility to CTL [46-50].

In summary, BNIP-2 is a pro-apoptotic granzyme B substrate and joins BID [5, 6] and MCL-1 [31] as molecules that interact with BCL2 family members and which are cleaved by caspases and granzyme B at distinct sites. Redundancy in the pro-apoptotic pathways exploited by cytotoxic lymphocytes maximises the chances of effective killing of infected cells or tumours and reduces the likelihood of evasion from apoptosis. However, such redundancy also makes functional analysis of individual components of this pathway a challenge.

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FIGURE LEGENDS

Figure 1

An IEAD motif in BNIP-2

Sequence comparison of the *N*-termini of BNIP-2 from multiple species. Asterisks are identical residues and the box shows conservation of the IEAD motif in mammals. Also shown is the percentage identity of each complete BNIP-2 molecule to the human BNIP-2 protein sequence. The alignment between human, chicken and puffer fish sequences reveals that the IEAD motif is not conserved beyond mammals. Accession numbers; Genbank: NM_004330.1 (Human); NP_001008239 (Mouse); NP_001100305 (Rat); XP_413765.1 (Chicken). Ensembl (www.ensembl.org): ENSMMUP00000026775 (Macaque), ENSPTRP00000012187 (Chimpanzee), ENSBTAP00000017939 (Cow); SINFRUP000000132680 (Puffer Fish), ENSMODP00000014559 (Opossum), ENSOCUP00000005429 (Rabbit).

Figure 2

Cleavage of target cell BNIP-2 during NK cell-mediated killing

(A) A cytotoxicity assay was performed using Daudi target cells stained with Cell Tracker Orange (CTO, y-axis) and NK effector cells stained with Cell Tracker Green (CTG, x-axis) at an effector:target ratio of 3:1. Cells were co-cultured for 2.5 or 4 hours before sorting (on the basis of CTG or CTO fluorescence). Each cell type was also cultured alone for 4 hours and sorted. The gating strategy used is indicated. The purity of the sorted cell populations ranged from 95–99% when analysed post-sort (figures were produced using FloJo software). The NK cells were inefficiently labelled with CTG and hence two populations of cells were detected based on CTG fluorescence. This experiment used the NK cell line NK92MI.

(B) Whole cell lysates of equivalent cell numbers of the sorted cell populations were analysed by SDS-PAGE and immunoblotting using antibodies against BNIP-2, PARP-1 and actin (as a loading control). Two species of BNIP-2 were detected in both the target and effector cells (labelled 1 and 2). For PARP-1, the 116 kDa species (labelled 3) and 89 kDa species (labelled 4) represent the full length and cleaved forms of PARP-1 respectively. This experiment used the NK cell line NK92MI.

Figure 3

Cleavage of recombinant BNIP-2 *in vitro*

(A) Domain structure of BNIP-2 protein showing the IEAD motif (amino acids 25–28), the granzyme B cleavage site (D²⁸, indicated by an arrow) and the C-terminal BNIP-2/CDC42 Homology (BCH) domain. A GST-BNIP-2myc fusion protein was constructed with a C-terminal myc epitope tag to facilitate detection. Site directed mutagenesis was used to change the aspartate (D²⁸) residue to an alanine (A²⁸).

(B) Cleavage of GST-BNIP-2myc at the D28 residue. *E. coli* expressed D²⁸ or A²⁸ derivatives were cleaved using lysates derived from YT (a human NK cell line), or IL-2 activated NK cells from peripheral blood (NK), or left untreated (-). The intact fusion protein (labelled BNIP-2) and cleaved species (denoted gBNIP-2 for cleavage by granzyme B) were detected via the C-terminal c-myc epitope tag.

(C) Cleavage is proportional to granzyme B expression. Lysates from the cell populations indicated were made at a constant cell density and used, either undiluted (x1), or at the dilutions shown to cleave BNIP-2/D²⁸. (0) is lysis buffer only. The inset shows granzyme B content of the lysates.

(D) GST-BNIP-2myc cleavage by recombinant granzyme B, the D²⁸ or A²⁸ derivatives were treated with 0, 8ng, 40ng or 200ng (approximately 12, 60 and 300nM respectively, increasing concentration indicated by the slope) of granzyme B for 10 minutes. A second cleavage site is present in BNIP-2 which was only cleaved at very high concentrations of recombinant granzyme B, this site is located close to the caspase cleavage site (data not shown).

Figure 4

Full length and truncated BNIP-2 induce PARP-1 cleavage

(A) HeLa cells were used untransfected (none), transfected with empty vector (vector) or with BNIP-2myc/D²⁸ or A²⁸ derivatives. Lysates were analysed for intact PARP and the cleaved form (cPARP), as a marker of apoptosis. Intact BNIP-2myc/D²⁸ or A²⁸ derivatives (BNIP-2) and the caspase cleaved form (cBNIP-2) are shown. Cleavage of PARP to cPARP and BNIP-2 to cBNIP-2 was caspase-dependent, as shown by zVAD-fmk inhibition. The band marked NS is a non-specific product and the locations of 47.5 and 32.5 kDa markers are indicated.

(B) Expression of full length BNIP-2myc/D²⁸ (BNIP-2) and an *N*-terminal truncation mutant (g)BNIP-2myc that mimics the granzyme B cleavage product, in HeLa cells, analysed at 6hr time intervals. Also shown are cells transfected with the vector only, mock transfected cells (M), and untransfected cells (-) analysed at the 24hr time point. The BNIP-2 and (g)BNIP-2 products were detected via the *C*-terminal myc epitope. PARP cleavage (cPARP) was analysed in response to BNIP-2 and (g)BNIP-2 expression as shown and actin used as a loading control. Also shown is a longer exposure of the (g)BNIP-2 blot, showing weak expression of this derivative. With full length BNIP-2, the caspase cleaved form (cBNIP-2) was visible at the 24hr time point whereas this species was not visible using (g)BNIP-2, presumably due to the low abundance (data not shown)

Figure 5

BNIP-2 is cleaved at the IEAD motif during NK cell-mediated killing

(A) BNIP-2 cleavage during NK cell-mediated killing. Left panel; HeLa cells transfected with BNIP-2myc/D²⁸ were co-cultured with NK cells for 0 or 5 hours in the presence or absence of 5 mM EGTA. EGTA inhibits NK cell-mediated killing by preventing the calcium-dependent polymerisation of perforin [16] and the transfer of granzyme B to the target cells (see also Figure 5B). Intact (BNIP-2) and granzyme cleaved BNIP-2 (gBNIP-2) are indicated. The input lanes on the left panel are the BNIP-2myc/D²⁸ transfected HeLa or NK cell lysate analysed in isolation. Centre panel shows the killing experiment performed with the A²⁸ derivative, EGTA treatment was not performed in this case since no cleavage was observed. The right hand panel shows lysates from A²⁸ transfected HeLa or NK cells alone. Size markers are shown. The caspase inhibitor zVAD-fmk was included in all cultures in this experiment.

(B) NK cell killing of HeLa is inhibited with EGTA. Cell Tracker Green labelled HeLa cells were co-cultured with NK cells for 0 or 5 hours in the presence or absence of EGTA and dead/dying cells identified by propidium iodide staining and flow cytometry. The numbers indicate the percentage of propidium iodide positive target cells. Events are gated on the Cell Tracker Green labelled cells to exclude NK cells from the analysis.

(C) EGTA treatment does not affect the BNIP-2 cleaving activity of NK lysates. Cleavage of GST-BNIP-2myc/D²⁸ was performed using NK cell lysates +/- 5mM

EGTA. These results show that granzyme B protease activity is unaffected by EGTA treatment. The input lanes show the fusion protein alone (D^{28}) or NK cell lysate alone.

(D) Granzyme B and caspase-mediated cleavage of BNIP-2 occurs at distinct sites. HeLa cells transfected with BNIP-2myc/ D^{28} were co-cultured with NK92 cells for 0 and 5 hours in the presence and absence of EGTA before analysis by immunoblotting with anti-myc antibody. Samples of BNIP-2myc/ D^{28} HeLa cell lysate in the presence and absence of EGTA and zVAD-fmk were analysed in parallel with the cytotoxicity assay. Untransfected HeLa and NK92 cells cultured alone were also included in the analysis. The locations of gBNIP-2 and cBNIP-2 together with size markers are indicated. NK92-XR indicates a cross-reacting species present in the NK92 cell lysate.

Figure 6

Target cell BNIP-2 and BID are dispensable for killing by human NK cells

(A) BNIP-2 protein expression in HeLa cells stably transfected with a vector expressing an shRNA targeting BNIP-2, an irrelevant shRNA (Control) or the empty vector. BNIP-2 expression was analysed in a pool of transfectants or individual clones as shown. BID expression was used as a loading control. Clones 2 and 4 exhibited a 75% reduction in BNIP-2 protein expression compared to the control shRNA transfectants.

(B) NK cell-mediated killing of HeLa cells lacking BNIP-2 expression (Clones 2 and 4) compared to killing of irrelevant shRNA transfected (Con) or vector only (Vec) transfected cells. The percentage of HeLa cells killed is indicated. The key indicates the effector (E) to target (T) ratios used. Each condition was analysed in triplicate in each experiment and the error bars show standard deviation from the mean based on three separate experiments.

(C). BID protein expression in HeLa cells transiently transfected with an irrelevant control siRNA (Control) or an siRNA targeting BID (as well as in mock transfected cells). Actin expression was used as a loading control. Expression of BID protein was reduced by a minimum of 80% compared to the control siRNA.

(D) NK cell-mediated killing of HeLa cells lacking BID expression (BID siRNA) compared to killing of control siRNA (Con) or mock transfected cells. The percentage of HeLa cells killed is indicated. Each condition was analysed in triplicate in each experiment and the error bars show standard deviation from the mean based on two separate experiments. The key indicates the effector (E) to target (T) ratios used in the killing assays.

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Figure 1

Human	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	ILAITGPEDQPGSLEVNGNKVRKKLMAPDISL...	100
Chimp	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	ILAITGPEDQPGSLEVNGNKVRKKLMAPDISL...	99
Macaque	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	ILAITGPEDQPGSLEVNGNKVRKKLMAPDISL...	99
Cow	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	ILAVTGPESQPGSLEVNGNKVRKKLMAPDISL...	98
Rabbit	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	VLDVAGPESRPGSLEVNGNKVRKKLMAPDISL...	96
Mouse	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	TLDGTDPRQPGSLEVNGNKVRKKLMAPDISL...	92
Rat	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	ILDVTDPRQPGSLEVNGNKVRKKLVAPDISL...	92
Opossum	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	ILTVAGPDHQPDPLEVNGNKVRKKLMAPDISL...	91
	*****	****	* * * *****	
Human	MEGVELKEEWQDEDFPIPLPEDDS	IEAD	ILAITGPEDQPGSLEVNGNKVRKKLMAPDISL...	100
Chicken	MEGVEFKEEWQDEDFPRPLPEDDP	VDSD	ILAAAGTE---GEIAVNGTKVRKKLTAPDISL...	88
Puffer Fish	MEGVELKEEWQDEDFPRPLPEEE-	ELED	ELFAGTSEGEPCYPAGQGKKAKKKLTAPNISL...	73
	*****		* * * * *	

Figure 2

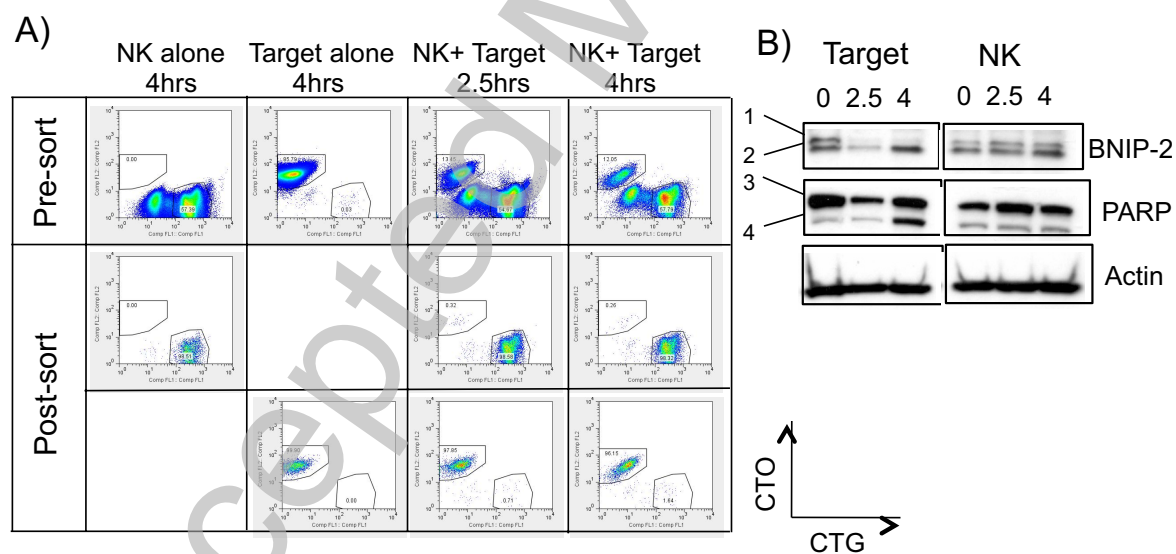


Figure 3

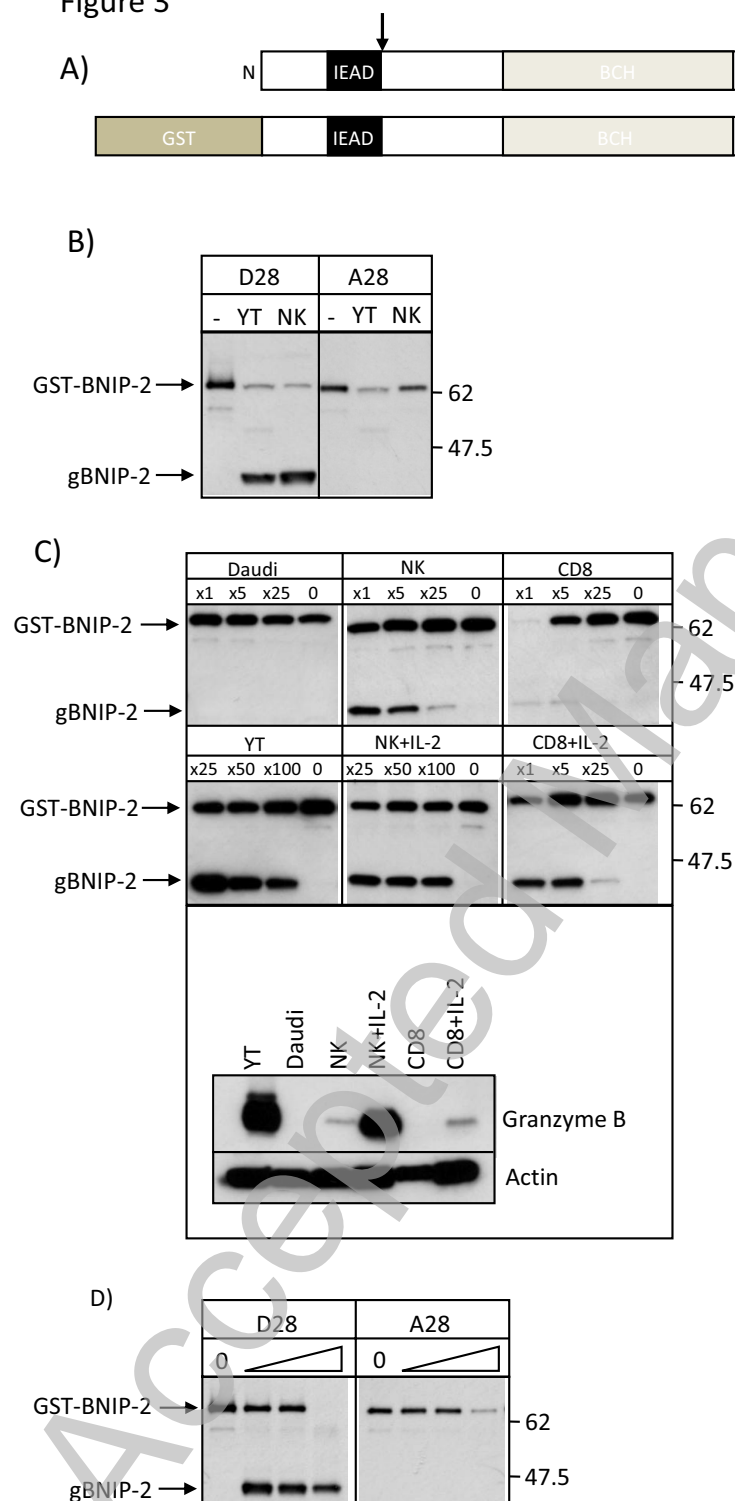
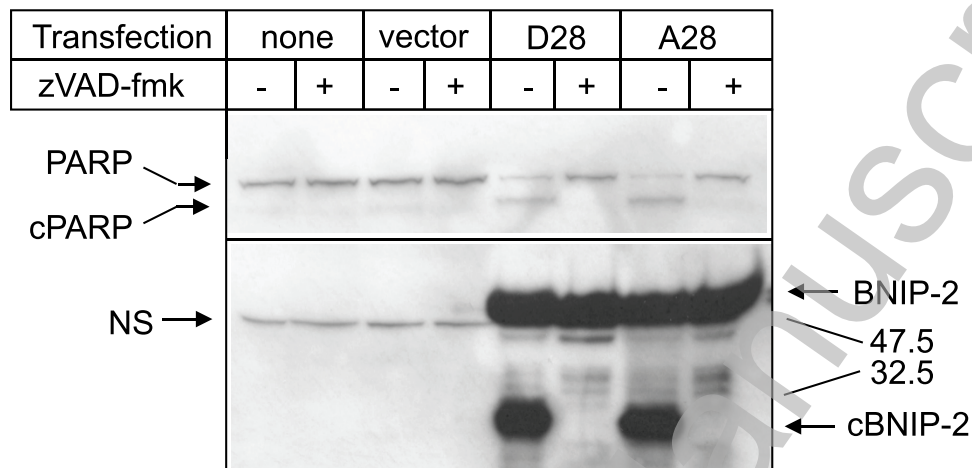


Figure 4

A)



B)

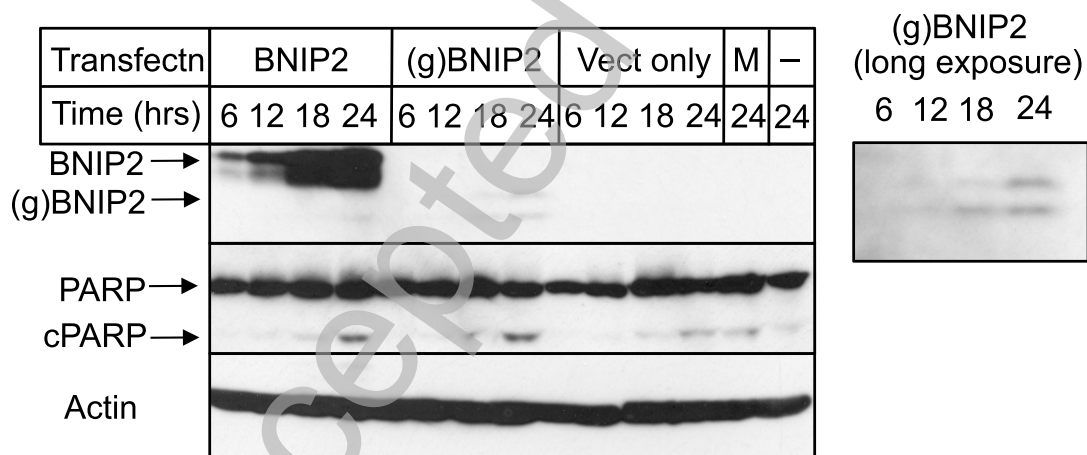
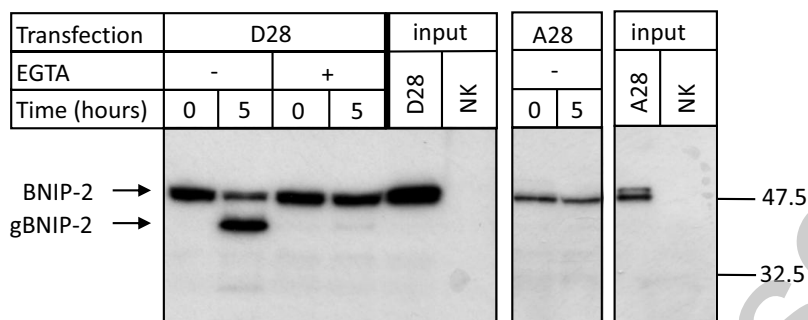
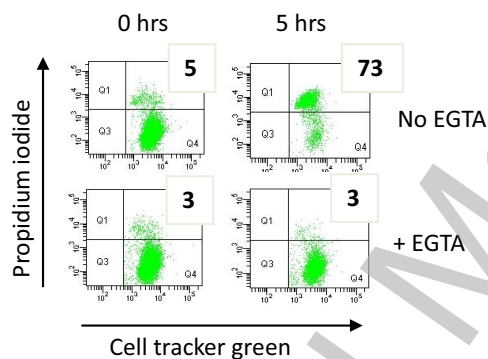


Figure 5

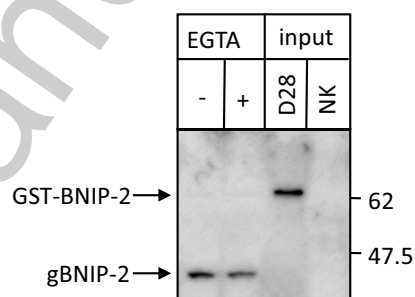
A)



B)



C)



D)



Figure 6

