Translocation of Full-Length Bid to mitochondria during Anoikis.

Anthony J. Valentijn and Andrew P. Gilmore

Wellcome Trust Centre for Cell Matrix Research
School of Biological Sciences
3.35 Stopford Building
Manchester University,
M13 9PT

Tel: 44 161 275 5576
Fax: 44 161 275 1501
Abstract.

Epithelial cells require adhesion to the extracellular matrix for survival, and in the absence of adhesion they undergo apoptosis (anoikis). This is distinct from apoptosis induced by extracellular death ligands, such as TNF, which result in direct activation of caspase 8. Bid is a member of the BH3-only subfamily of the Bcl-2 proteins, and is important for most cell types to apoptose in response to Fas and TNF-R activation. Caspase 8 cleaves full-length Bid (FL-Bid), resulting in truncated p15 tBid. p15 tBid is potently apoptotic, and activates the multidomain Bcl-2 protein, Bax, resulting in release of cytochrome c from mitochondria. We have previously shown that Bax rapidly translocates from the cytosol to mitochondria following loss of adhesion, and that this is required for anoikis. We have now examined the role of Bid in anoikis. Bid translocates to mitochondria with identical kinetics as Bax. Although Bid is required for anoikis, it does not require proteolytic cleavage by caspase 8. Furthermore, it does not require Bid to interact directly with other Bcl-2 family proteins, such as Bax. Our data indicate that Bid is important for regulating apoptosis via the intrinsic pathway, and has implications for how Bid may fulfil that role.

Keywords

Anoikis, Bid, Bax, caspase 8, extracellular matrix
Introduction.

The Bcl-2 family of proteins are important regulators of programmed cell death (1). The family consists of members that are either anti-apoptotic (e.g. Bcl-2 and Bcl-X<sub>L</sub>) or pro-apoptotic (e.g. Bax and Bak), and are characterised by sharing up to four Bcl-2 Homology (BH) domains. The anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> share BH-domains 1-4, whereas pro-apoptotic Bax and Bak have only domains 1-3. The exact mechanism by which these molecules induce apoptosis is still unclear, but they appear to regulate permeabilisation of the outer mitochondrial membrane (OMM), causing release of apoptogenic factors such as cytochrome <em>c</em> (2,3). Pro-apoptotic Bax and Bak are absolutely essential for this, and cells deficient in both of these are resistant to a wide variety of cellular insults (4). A subset of pro-apoptotic Bcl-2 proteins have only the BH3 domain, and are referred to as the BH3-only proteins (5). BH3-only proteins sense diverse death stimuli and regulate the activity of multidomain Bcl-2 proteins, such as Bax. For example, Bim is regulated at a transcriptional level downstream of cytokine and growth factor receptors, whereas NOXA and PUMA are expressed in response to p53 activation (6-8). Others are regulated post-translationally, such as Bad, which is phosphorylated downstream of growth factor receptors, sequestering it in the cytosol through interactions with 14-3-3 proteins (9).

Bid, another BH3-only protein has a role in apoptosis initiated by the extrinsic pathway (10). Engagement of death receptors (e.g. Fas or TNF receptor) results in activation of caspase 8, which cleaves Bid at D59 to produce a truncated Bid (tBid) (11). Inhibition of Bid cleavage blocks apoptosis in response to death receptor activation in most cells(11,12). tBid translocates to mitochondria where it mediates cytochrome <em>c</em> release through activation of Bax and Bak. Cells deficient in Bax and Bak are resistant to tBid induced apoptosis (4). tBid has been shown <em>in vitro</em> to induce a conformational change in Bax followed by its oligomerisation and insertion into the OMM (13-15). Therefore, activation of Bid may be one mechanism by which Bax is induced to initiate OMM permeabilisation.
In this paper we have examined the role of Bid in apoptosis mediated by the intrinsic pathway. Epithelial cells depend on interactions with the extracellular matrix (ECM) via integrins for survival, and detachment from ECM results in apoptosis referred to as anoikis (16). Anoikis is required to ensure cells only survive within their proper context in a particular tissue. Adhesion dependent kinases provide a strong survival signal, although different cell types utilise different downstream pathways (17-19). Signalling molecules implicated include pp125FAK, PI3-kinase, JNK and MEK. A number of BH3-only proteins are regulated by these pathways, including Bad, Bim and Bid. Bim has been suggested to be involved in anoikis in MCF10A cells (20). Expression of Bim increased over 24 to 48 hours following detachment from ECM, concomitant with the down regulation of epidermal growth factor receptor (EGFR). Indeed, Bim appears to be more closely regulated by growth factor signalling than other forms of cellular insults, and it is not clear if Bim was directly regulated by adhesion signals (6,21,22). Translocation of Bax to mitochondria occurs within 15-30 minutes of detachment, too rapid to be explained by the upregulation of Bim expression. Furthermore, anoikis does not require new protein synthesis, indicating that proapoptotic proteins which are transcriptionally regulated are unlikely to have a central role in this process (23). A number of studies have implicated death receptor activation and subsequent activation of caspase 8 as an initiating event for anoikis (24,25). Detachment from ECM also leads to an increase in Fas expression on endothelial cells, making them sensitive to FasL (26). Alternatively, in adherent endothelial cells, unligated integrins can directly activate caspase 8 (27). We have shown that anoikis of epithelial cells does not require activation of caspase 8, and that translocation of Bax, its N-terminal conformational change and cytochrome c release, all occur in a caspase independent manner (17,23,28).

We have now studied the subcellular localisation of Bid during anoikis of mammary epithelial cells. We show that Bid rapidly translocates to mitochondria following cell detachment from ECM, and that this occurs in the absence of proteolytic cleavage. Full length (FL) Bid in which the caspase 8 cleavage site was mutated (D59E BID) translocates to mitochondria in an identical manner as WT Bid. Furthermore, translocation of Bid appears to be required for anoikis, but does not require a direct
interaction with other Bcl-2 proteins on the OMM. Together, our data suggest that endogenous FL-Bid can be activated into a pro-apoptotic form in the absence of caspase cleavage, and that this may have a role in the intrinsic cell death pathway.
Materials and methods

Reagents
Polyclonal goat anti-Bid, rabbit anti-Bad and recombinant tBid were from R&D Systems. Rabbit anti-actin and anti-Bak were from Sigma. Rabbit anti-Bcl-X\textsubscript{L} was from BD Biosciences. Rabbit anti-GFP was from Molecular Probes. Monoclonal anti-V5 was from Invitrogen. Monoclonal anti-mtHsp70 and anti-APAF1 were obtained from Affinity Bioreagents. The caspase inhibitors zVAD-fmk and IETD were from CN Biosciences. Rabbit recombinant caspase 8 was from CN Biosciences. Poly-HEMA was from Sigma. Secondary antibodies (anti-rabbit, anti-goat and anti-mouse peroxidase conjugates, and anti-mouse Cy3 conjugates) were from Stratech Scientific.

Expression constructs.

A murine Bid cDNA was amplified from a mouse embryo cDNA library with Pfu polymerase (Promega) using oligonucleotide primers to the 5’ and 3’ ends of the coding sequence, cloned into pCRScript (Stratagene), and confirmed by sequencing. To generate GFP-Bid and Bid-GFP, PCR primers were used that incorporated restriction endonuclease sites, and the product was ligated into either pEGFP-C3 or pEGFP-N1 (Clontech). pEYFP-Bax, pECFP-BclX\textsubscript{L}, pHcReD-XT and pEYFP-XT have all been previously described (28). All constructs were confirmed by DNA sequencing and immunoblotting. To express V5 tagged Bid, Bid was subcloned into pCDNA6-V5-His (Invitrogen). The single amino acid substitutions were generated using the Quick Change mutagenesis kit (Stratagene) according to the manufactures instructions. Bid deletions were made using PCR.

Cell culture and transfection.

FSK-7 cells were cultured and transfected as previously described (17). Briefly, cells were plated at 1x10\textsuperscript{5} cells/cm\textsuperscript{2} on glass cover slips 18 hours prior to transfecting with 0.5 \(\mu\)g DNA in a 12-well tissue culture plate using Lipofectamine Plus (Life Technologies).
18 hours post transfection, cells were washed extensively with PBS. For detachment assays, cells were trypsinised and replated onto 35 mm dishes coated with poly-HEMA (Sigma). Cells maintained on poly-HEMA were collected by centrifugation (5000 x g, 30 seconds). For immunostaining, cells were resuspended in culture medium and centrifuged onto polysine slides (Merck) using a Cytospin cytological centrifuge (Shandon). For fractionation studies, cells were resuspended into hypotonic buffer (see below). Adherent cells were scraped into hypotonic buffer.

**siRNA.**

Cells were transiently transfected as above with pSilencer 1.0U6 (Ambion) containing either a target sequence against bases 79-87 of the murine Bid coding sequence, or a control sequence with an equivalent base content. Empty pSilencer was used as a further control. Cells were co-transfected with pEGFP.C3. Cells were cultured for 48 hours post transfection. For immunoblotting, cells were trypsinised, washed extensively in serum free DMEM/F12, and sorted using a BD Biosciences FACS Vantage, using a 488nm Argon laser. Collected cells were lysed into SDS-PAGE sample buffer. For apoptosis assays, cells were trypsinised and plated onto poly-HEMA coated dishes for 5 hours. They were then cytopspun onto polysine slides (Merck), fixed in 4% formaldehyde/PBS, and nuclei stained with Hoescht. Apoptosis was quantified in GFP positive cells on the basis of nuclear morphology.

**Caspase 8 cleavage of V5 epitope tagged Bid.**

FSK-7 cells were transfected as above, except with 1µg of DNA on 50mm tissue culture dishes. Cells were harvested 18 h post transfection by scraping into ice cold caspase digestion buffer (50mM HEPES-Cl, pH 7.5, 50mM NaCl, 0.1% CHAPS, 10mM EDTA, 5% glycerol). Lysates were cleared by centrifugation at 20000xg for 20 min at 4°C, and protein concentration was estimated using the BCA protein assay (Pierce). 500 µg of cell lysate was immunoprecipitated with 1µg of monoclonal anti-V5 and protein G sepharose (Roche) for three hours at 4°C. After washing three times with digestion buffer, the
immunocomplex was resuspended in 40µl of the same buffer to which DTT was added to 10 mM. Human recombinant caspase 8 (1.5 units) was added and incubated for 2.5 h at 37°C. Reactions were stopped by adding SDS-PAGE sample buffer and boiling. Samples were separated by SDS-PAGE, electroblotted to nitrocellulose and immunoblotted for Bid.

**Subcellular fractionation.**

Adherent cells were scraped and detached cells collected by centrifugation (1200xg, 3 mins). Following several washes in ice cold PBS containing 1mM Na3VO4, 4mM NaF, cells were resuspended in hypotonic lysis buffer (10mM HEPES-Cl, pH 7.5, 10mM NaCl, 1.5mM MgCl2) supplemented with 1mM Na3VO4, 4mM NaF and protease inhibitor cocktail (Calbiochem), and Dounce homogenised on ice. NaCl was then added to a final concentration of 150mM. Lysates were centrifuged at 100000 x g for 30 min at 4°C. Supernatant (s fraction) was saved and the pellet (m fraction) was resuspended in an equal volume of 0.25% CHAPS/10 mM HEPES-Cl, pH7.5, 150 mM NaCl. After brief sonication, the CHAPS extracted pellet was centrifuged as before and the supernatant saved. Protein concentration of the s fraction was determined using the BCA assay to normalise between samples. To compare soluble and membrane associated proteins, equivalent amounts of s and m fractions were analysed.

**Cell fractionation and chromatography.**

For gel filtration of CHAPS extracted membrane fractions, 5 mg of protein in a volume of 0.5 ml was loaded onto a Sephacryl S100-HR column (1.5 x 25 cm, Pharmacia, equilibrated in 10 mM HEPES-Cl pH7.6/150 mM NaCl/4 mM CHAPS). The column was resolved at 4°C at a flow rate of 0.1 ml/min. 0.8 ml fractions were collected. Calibration was with molecular weight standards from Sigma (12.4 kDa, cytochrome c; 29 kDa, carbonic anhydrase; 66 kDa, bovine serum albumin; 200 kDa, β-amylase; void volume, blue dextran). Column fractions were concentrated with TCA, separated by reducing SDS-PAGE, transferred to nitrocellulose (Biorad), and immunoblotted.
**Crosslinking.**

Following hypotonic lysis, samples were incubated with 0.5 mM of the Homobifunctional reagent BS3 (Pierce) for 30 minutes on ice. Reactions were quenched with 50mM Tris-Cl, pH7.6, and fractionated as above. Crosslinked soluble and membrane fractions were then separated by SDS-PAGE and immunoblotted.

**Fluorescence microscopy.**

Cells were fixed in 4% formaldehyde/PBS. Cells expressing GFP and HcRed tagged constructs were then mounted in ProLong (Molecular Probes) without further manipulation. Cells requiring immunostaining were permeabilised in PBS/0.5% Triton-X100. Primary antibodies were diluted in PBS/0.1% Triton-X100/0.1% horse serum (1 hour, 37°C). Following washing in PBS, secondary anti-mouse Cy3 conjugates were incubated in above buffer (30 minutes, 37°C). Cells were counterstained with 1 µg/ml Hoescht 33258. Coverslips were mounted in ProLong. Most images were collected on an Olympus IX70 inverted microscope using a 100 x PLAN-APo 1.4NA objective, equipped with a DeltaVision imaging system. Images were processed by constrained iterative deconvolution on softWoRx V3.0 (Applied Precision). CFP and GFP co-expressing cells were viewed on an Leica SP2-AOBS confocal in order to separate the overlapping fluorophores. CFP was excited at 405nm (>10% GFP excitation) using a blue diode laser and viewed at 425-450nm. GFP was excited at 476nm (>10% CFP excitation) using an argon laser, and viewed at 530-575nm.
Results

Full-length Bid translocates to mitochondria rapidly during anoikis.

We have previously shown that mammary epithelial cells require adhesion to ECM for survival, and that following detachment Bax translocates to mitochondria (17,28). BH3-only proteins function upstream to activate the multidomain proteins such as Bax. We therefore asked which BH3-only proteins become activated in response to cell detachment from ECM. FSK-7 mammary epithelial cells were detached from the ECM for various times before separating into cytosolic and membrane fractions. We found that Bid rapidly translocated to the membrane fraction following detachment (Fig 1a). A small amount of Bid was detectable in the membrane fraction of adherent cells, but this significantly increased within 15 minutes of removing cells from ECM. Bid translocation preceded the detection of active caspase 3 in the cytosol. Fractions were immunoblotted for other Bcl-2 family proteins (Fig. 1b). As previously described, Bax also translocates to the membrane within 15 minutes (17). Conversely, Bcl-XL and Bak are constitutively associated with the membrane fraction. The BH3-only protein, Bad, also did not show any significant redistribution following detachment (Fig. 1b). Bad is distributed between the membrane and cytosolic fraction. Translocation of Bid exclusively involves FL-Bid (Fig. 1c). At no point during anoikis did we observe significant amounts of 15 kDa tBid, either in the cytosol or membrane fractions. Prolonged exposure of the immunoblots clearly show that the vast majority of membrane associated Bid is full length. The size of tBid is indicated by the presence of 1ng of recombinant tBid.

To confirm that FL-Bid associates with mitochondria, we transiently expressed in cells Bid tagged with GFP, either at the C-terminus (Bid-GFP), or the N-terminus (GFP-Bid) (Fig 2a). Subcellular localisation was determined by expression of HcRed-XT, an OMM marker consisting of the C-terminal domain of Bcl-XL (28,29). In adherent cells, both GFP-Bid and Bid-GFP show a diffuse, cytosolic distribution (Fig. 2b). However, following detachment, both forms of tagged Bid extensively colocalise with HcRed-XT. There is no co-localisation between GFP and HcRed-XT in adherent or detached cells.
Together, these data indicate that FL-Bid translocates rapidly to mitochondria following the detachment of cells from ECM.

**Bid translocates independently of caspase activation.**

Our recent data indicates that anoikis does not require activation of caspase 8. Inhibiting caspase 8 did not inhibit Bax translocation or the release of cytochrome c during anoikis (23). However, some studies have suggested a role for death receptors and caspase 8 activation as an initiating event following cell detachment (24,25). Given these conflicting data, we carried out a number of experiments to confirm that Bid translocation is independent of proteolytic cleavage. Cells were pretreated with either zVAD or caspase 8 specific IETD for 15 minutes before they were detached from ECM. We have previously shown that the concentration of zVAD used here inhibits anoikis in FSK-7 cells, although it does not prevent Bax translocation or cytochrom c release (23). Cytosolic and membrane fractions were isolated and immunoblotted for endogenous Bid (Fig. 3a). Neither caspase inhibitor prevented the translocation of endogenous Bid to the membrane fraction. Next, we made the conservative amino acid substitution D59E. Substitution of this site to either glutamate or alanine blocks caspase 8 cleavage (11,30). WT-Bid and D59E-Bid were expressed with a C-terminal V5-epitope tag (BidV5), distinguishing them from endogenous Bid when transiently expressed in FSK-7 cells (Fig. 3b). D59E-Bid was resistant to cleavage by recombinant caspase 8 in vitro (Fig. 3b). We compared the ability of D59E and WT-BidV5 to translocate to mitochondria during anoikis. Transiently transfected cells were detached from ECM and cytosolic and membrane fractions isolated. These were immunoblotted for Bid (Fig. 3c). Both WT and D59E BidV5 translocate to the membrane fraction concomitant with endogenous Bid in the same samples. We examined the subcellular localisation of WT and D59E BidV5 in cells co-expressing YFP-XT to mark the OMM (Fig. 3d). Both forms of BidV5 were cytosolic in adherent cells. Following detachment, both extensively colocalised with YFP-XT.
Bid regulates anoikis in mammary epithelial cells and translocates independently of Bax.

We wished to determine if FL-Bid was required for anoikis. A non-cleavable mutant form of Bid sensitizes cells to a variety of apoptotic stimuli when overexpressed in mouse embryonic fibroblasts (30). We asked if transfection of either WT Bid-V5 or D59E Bid-V5 sensitises epithelial cells to anoikis. Cells transiently expressing either form of Bid were detached from ECM for 5 hours. Apoptosis in V5 positive cells was quantified by nuclear morphology and compared with cells transfected with control vector (Fig. 4a). There was a small increase in apoptosis in adherent cells transfected with either form of Bid compared with control cells. However, detachment for 5 hours indicated that both WT and D59E BidV5 sensitised cells to anoikis. There was no difference between WT Bid or D59E BidV5 in the degree of sensitisation. We next downregulated endogenous Bid using siRNA. FSK-7 cells were transfected with either control pSilencer or pSilencer expressing siRNA targetted to murine Bid. Cells were co-transfected with pEGFP. 48 hours post transfection cells were either sorted by FACS for western analysis (Fig. 4b) or detached from ECM and maintained on poly-HEMA for 5 hours. Cells in which Bid expression had been knocked down showed a much reduced level of apoptosis following detachment compared with cells transfected with control pSilencer (Fig. 4c).

Overexpression of Bcl-XL or Bcl-2 can block activation and translocation of Bax (31,32). We asked if expression of Bcl-XL blocked the translocation of Bid during anoikis and its ability to sensitize cells to apoptosis. Cells co-expressing Bax, WTBidV5 or D59EBidV5, along with either GFP or GFP-Bcl-XL, were detached for 5 hours and apoptosis quantified by nuclear morphology (Fig.5a). GFP-Bcl-XL inhibited sensitisation to anoikis due to overexpression of Bid or Bax. We then asked if this was due to inhibition of Bid translocation. Cells were co-transfected with YFP-Bax or Bid-GFP, with or without CFP-Bcl-XL. Cells were detached from ECM for 30 minutes and the localisation of each determined (Fig.5b). In the absence of CFP-Bcl-XL, both YFP-Bax and Bid-GFP showed a punctate distribution in detached cells, distinct from the cytosolic distribution seen in adherent cells. However, although CFP-Bcl-XL inhibited the translocation of YFP-Bax, it
did not block the redistribution of Bid-GFP. These data indicate that Bid translocation is
independent of Bax relocalisation to mitochondria. Both Bid and Bax are required,
however, as our previous data from Bax null epithelial cells, which show delayed anoikis,
indicates (28). These cell express normal levels of Bid (unpublished data).

**The ability of Bid to regulate anoikis does not require a direct interaction with other
Bcl-2 family proteins.**

Bid can bind to a number of other Bcl-2 proteins, including Bax and Bcl-2 (33). However, although Bax enters large mitochondrial associated clusters following OMM permeabilisation, Bid does not (34). Bid can activate Bax, both *in vitro* and *in vivo*, but it remains unclear when, or indeed if, they interact. Most Bcl-2 proteins associate with other mitochondrial components, and form stable complexes which can be isolated by gel filtration. Bax translocates to mitochondria as a monomer before forming stable oligomeric complexes (35-37). We therefore asked if Bid associated with other mitochondrial proteins following translocation to mitochondria, and whether we could detect it in fractions which contained Bax.

Soluble and membrane fractions were prepared from FSK-7 cells either in monolayer or
maintained on poly-HEMA. The membrane fractions were then further extracted in
CHAPS, and the CHAPS insoluble material solubilised in SDS-PAGE sample buffer. All
three fractions were analysed by SDS-PAGE and immunoblotting for Bax and Bid (Fig.
6a). Bax becomes associated with a CHAPS insoluble fraction during anoikis (28). This
did not occur with Bid, which was completely extracted by CHAPS at all stages of
anoikis. The CHAPS soluble fractions were subjected to S100 chromatography, separated
by SDS-PAGE and immunoblotted for a variety of Bcl-2 family proteins (Fig.6b). As
previously shown, Bax associates with mitochondria initially as a monomer, subsequently
entering a high molecular weight fraction. In contrast, Bcl-Xₐ, which is constitutively
associated with mitochondria, elutes in high molecular weight fractions in both adherent
and detached cells. Other proapoptotic Bcl-2 proteins (Bak and Bad) were also
constitutively associated in complexes, with no monomeric forms detected on the OMM.
Cytosolic Bad coeluted with 14.3.3 proteins (our unpublished data). We found that FL-Bid, like Bax, translocates to mitochondria as a monomer. However, unlike Bax it does not enter a high molecular weight fraction. Indeed, of all the Bcl-2 family proteins we assayed, Bid was the only one we could not detect in a higher molecular weight complex. Possible interactions between Bid and other proteins may be extremely labile or sensitive to CHAPS, and thus not detected by our gel filtration assay. To address this we isolated membrane fractions and immediately crosslinked them with the homobifunctional reagent BS3. Murine Bid contains 7 lysine residues, which are distributed over the surface of the folded polypeptide (38). These were then separated by SDS-PAGE and immunoblotted for Bid (Fig.6c). Bid runs with a slightly slower mobility following crosslinking. However, no difference was seen between cytosolic Bid isolated from adherent cells and membrane associated Bid from cells undergoing anoikis. This is in contrast to our previous data with crosslinked mitochondrial Bax during anoikis (28).

Bid can associate with other Bcl-2 proteins via its BH3 domain (33). As mitochondrial Bid is exclusively monomeric, we asked if the BH3 domain is required for FL-Bid translocation. We expressed a series of Bid mutants in FSK-7 cells (Fig.6d). p15Bid constitutively interacts with mitochondria and activates Bax and Bak. We engineered a further deletion, consisting of the C-terminal 5 α-helices of Bid, but which does not contain the BH3 domain (p11BidV5). When expressed in FSK-7 cells, p15BidV5 was constitutively apoptotic (Fig.6e). p11BidV5 did not show any pro-apoptotic effect. We examined the subcellular distribution of both p15BidV5 and p11BidV5. p15Bid expressing cells had to be treated with 100µM zVADfmk in order to observe any adherent cells. Both forms of truncated Bid showed constitutive localisation with coexpressed YFP-XT, compared with WT BidV5, which was distributed throughout the cytosol (Fig.6f).

To ask if an interaction with other Bcl-2 family proteins was required for Bid translocation, we transfected cells with two forms of BidV5 containing single amino acid substitutions within the BH3-domain (Fig.6c). These substitutions inhibit Bid from interacting with other Bcl-2 proteins (33,39). mIII3 cannot interact with Bax, but still
associates with Bcl-2, whereas mIII4 does not interact with Bax, Bak or Bcl-2. Both BH3 domain mutants translocated to mitochondria with identical kinetics as WTBid-V5 (Fig. 6g). Furthermore, both mutants sensitised cells to anoikis to an identical extent as WT BidV5 (Fig.6e). Indeed, we have been unable to demonstrate any difference in the ability of mIII3, mIII4 and WT BidV5 to induce apoptosis. These data show that the interaction of Bid with the OMM during apoptosis is not dependent upon its ability to interact with other Bcl-2 proteins. Furthermore, they suggest that although Bid requires Bax and/or Bak to induce apoptosis, it does not require any direct interaction with them.

**Regulation of FL-Bid translocation during anoikis by pp125FAK and PI3-kinase.**

Given that Bid and Bax translocated independently, we wished to determine if similar integrin mediated signals were regulating them. Specific signalling pathways downstream of adhesion regulate anoikis and Bax translocation (17-19). Of these, pp125FAK and PI3 kinase have been implicated in a number of studies, and inhibition of either kinase can induce Bax translocation (17). Protein kinase B, a downstream effector of PI3-kinase, also regulates Bax function (40). PI3-kinase also inhibits Fas mediated apoptosis following T-cell activation, suggesting a role in regulating Bid function. To determine if Bid was regulated by signalling enzymes known to suppress anoikis and Bax function, we utilised dominant negative forms of both pp125FAK and PI3-kinase. We co-transfected FSK-7 cells with either WT or D59E BidV5, along with pCDNA3, pCDNA3 expressing dominant negative PI3-kinase (DN-p85) or dominant negative pp125FAK (DN-FAK). Cells transfected with pCDNA3 showed a predominantly cytosolic localisation of both WT and D59E Bid (Fig. 7). However, in cells expressing either DN-p85 or DN-FAK, both WT and D59E BidV5 showed extensive co-localisation with mitochondria. These data suggest that survival signalling pathways which regulate Bax also control Bid at the level of its translocation to the OMM. It will be important to now determine how these pathways regulate Bid, and if it is through a direct or indirect mechanism.
Discussion.

In this paper we demonstrate that Bid has a general role in apoptosis regulated via the intrinsic pathway, and that this occurs independently of its cleavage by caspases. Furthermore, we demonstrate that the ability of FL-Bid to induce apoptosis through the intrinsic pathway does not require any direct interaction with other Bcl-2 family proteins on the OMM. Specifically, we show that endogenous Bid is regulated by a physiological apoptotic signal (anoikis) independent of caspase 8 cleavage. Translocation of endogenous FL-Bid to mitochondria occurs in the presence of both the caspase 8 specific inhibitor IETD, and the pan-caspase inhibitor zVAD. Both WT Bid and an expressed non-cleavable form of Bid (D59E-Bid) translocate to mitochondria within 15 minutes following detachment of epithelial cells from ECM. Downregulation of endogenous Bid with siRNA reduced cell sensitivity to anoikis. Conversely, overexpression of either WT or non-cleavable Bid sensitises cells to detachment induced apoptosis.

Previous results that have suggested a role for Bid in apoptosis initiated independent of death receptor activation have proposed that it functions as an amplification factor following OMM permeabilisation. In this scenario, generation of tBid by caspase 3 would result in further activation of Bax and Bak (41). Such a model requires that there is an initial, small release of cytochrome c, followed by some caspase activation and Bid cleavage, followed by catastrophic OMM disruption and cell death. Several lines of evidence suggest that this is not the case. OMM permeabilisation and caspase activation are rapid and complete events (28,42-44). No gradual leakage occurs and thus no amplification is required. Furthermore, in the present study we show that translocation of Bid occurs independent of proteolytic cleavage. This suggests that any cleavage of Bid occurs after cells have irreversibly committed to die. During anoikis we saw no evidence that significant amounts of endogenous Bid were cleaved even after caspase 3 activation.

To date there has been little evidence that endogenous FL-Bid functions as an apoptotic regulator for intrinsic cell death pathways. Our data now provides this evidence. Furthermore, both expressed caspase 8 resistant Bid and WT Bid translocate to
mitochondria following detachment from ECM within 15 minutes, as does endogenous Bid. Both forms also sensitised cells to anoikis to an identical extent. Not only does this suggest that FL-Bid functions upstream of mitochondrial permeabilisation, but it also indicates that an amplification loop involving Bid cleavage is not required. Down regulation by siRNA indicates that endogenous Bid is required for anoikis to occur normally. Interestingly, we did not see an inhibition of Bax translocation in cells expressing Bid siRNA (our unpublished data).

Our results suggest that FL-Bid is pro-apoptotic. There is growing evidence that this is the case and that cleavage by caspase 8 is not an obligate step for Bid activation. FL-Bid has always been seen to have apoptotic activity when overexpressed, and can induce insertion and oligomerisation of Bax into the OMM in vitro (13). FL-Bid translocates to mitochondria in HeLa cells treated with staurosporine, as well as in Jurkat cells exposed to anti-Fas antibodies in the presence of caspase 8 inhibitors. A recent report has demonstrated that a non-cleavable mutant of Bid (D59A) is potently apoptotic in mouse embryonic fibroblasts (MEFs) (30). Indeed, not only was this caspase resistant Bid as good at sensitising cells to apoptosis as over expressed WT Bid, but WT Bid was more similar to the D59A mutant in its apoptotic activity than it was to p15tBid. Such data suggests that the expressed p15 fragment of tBid does not provide a true representation of how WT Bid induces apoptosis.

How does FL-Bid function? Exogenously expressed p15 tBid is 10 times as effective at inducing cytochrome c release compared with FL-Bid, and can directly induce OMM permeabilisation independent of other Bcl-2 proteins (15,39). However, caspase 8 cleaved Bid is a heterodimer of both the resulting N-terminal and C-terminal fragments, and not a truncated form of the protein such as expressed tBid. Unlike p15tBid, heterodimeric cleaved Bid does not permeabilise mitochondria in the absence of Bax, even at very high concentrations (15). Previously, it had been assumed that caspase 8 cleavage resulted in dramatic conformational changes, an idea supported by the potent pro-apoptotic nature of p15tBid. (45). NMR studies now show that caspase 8 cleavage of FL-Bid does not result in a dramatic conformational change (38,46). If caspase cleavage
results in only subtle changes in Bid conformation, then these could also be brought about by mechanisms other than proteolysis. Although we still do not know how FL-Bid is induced to translocate to mitochondria, it is not necessary to invoke a radically different mechanism to that following caspase 8 cleavage.

It is still unclear if Bid is directly modified by ECM dependent signalling pathways. We have partially linked FL-Bid activation to kinase signalling pathways downstream of cell adhesion, specifically PI3-kinase. Expression of a dominant negative p85 subunit of PI3-kinase induced Bid translocation. Similarly, a dominant negative Focal Adhesion Kinase (FAK) also induced Bid translocation. FAK activates PI3-kinase following cell adhesion to ECM (47). PI3-kinase has been demonstrated to regulate Bid activation downstream of death receptor signalling via protein kinase B, as well as in response to IL3 withdrawal in FL5.12 cells (40,48,49). Bid can be phosphorylated on a number of serine and threonine residues within an unstructured loop between $\alpha$-helices 2 and 3. This region contains the sites for caspase 8 and granzyme B, and phosphorylation alters sensitivity to proteolytic cleavage (50,51). Substitution of any or all of these sites did not alter the ability of FL-Bid to translocate to mitochondria during anoikis, or affect its ability to sensitise cells to apoptosis (our unpublished data). At present we have not been able to identify a post-translational modification on Bid that occurs in response to adhesion signalling, and it is more than possible that any effect on Bid may be indirect.

So what targets Bid to mitochondria and how does it function there? As previously shown for expressed p15 tBid (33,52), FL-Bid does not require interactions with other Bcl-2 proteins to target to the OMM. Single amino acid substitutions within the BH3 domain, which abolish interactions with other Bcl-2 proteins, do not block its translocation to mitochondria. In fact, Bid does not appear to stably interact with any other proteins on the OMM, as it appears to be monomeric both before and after translocation. One report has indicated that p15tBid forms complexes on the OMM (39). We found no evidence that FL-Bid forms a stable interaction on the OMM during anoikis, and remains a monomer that is easily extracted with CHAPS. We cannot totally exclude the possibility that Bid forms interactions that are sensitive to CHAPS. However, we were also unable to
crosslink Bid into any higher molecular weight complex using BS3. In contrast, Bax translocates as a monomer, and then assembles into complexes which become resistant to CHAPS extraction. Bad is only found in stable complexes on the OMM, as are the multidomain proteins Bcl-Xₐ and Bak. Bid targeting may be mediated by interactions with lipids. Changes in the lipid composition of mitochondria have been associated with Fas mediated apoptosis (53). Bid interacts with the mitochondrial specific lipid cardiolipin and its metabolites, and cardiolipin is required for Bid to cause Bax dependent OMM permeabilisation (15,53,54). Studies by a number of groups have defined a region within the C-terminus required for this interaction. Expression of a C-terminal p11 fragment of Bid containing the lipid binding site in adherent FSK-7 cells indicates that this domain targets to the OMM constitutively, in agreement with previous reports (52). This might suggest that Bid targeting is not brought about by changes on the OMM itself, but instead might involve a conformational change in Bid to expose the lipid binding domain. It will be important to see if changes in the lipid composition of the OMM occur during anoikis, and if they occur prior to mitochondrial permeabilisation.

Our data indicates that Bid can induce apoptosis independent of its ability to interact with other Bcl-2 proteins. Our inability to detect an interaction with Bid by crosslinking or gel filtration may have been due to it being extremely transient. However, amino acid substitutions within the BH3 domain that block its interaction with Bax do not inhibit it from sensitising cells to anoikis. These substitutions have previously been shown not to inhibit the ability of p15 tBid to induce membrane permeabilisation, even though they abolish it from binding pro- and anti-apoptotic Bcl-2 proteins (39). Further support for this comes from a recent report showing that the ability of Bcl-Xₐ to inhibit Bim activation of Bax is not dependent on any of these proteins directly interacting with each other, suggesting that models in which BH3 domains interact with surface grooves in Bax and Bcl-Xₐ may not provide the complete story. BH3 only proteins may alter the lipid environment on the OMM to facilitate the function of multidomain molecules such as Bax and Bak (10).
Apoptosis is the end result of a cell interpreting multiple survival signals. Our data suggest that an early response to detachment in adhesion dependent cells is the non-proteolytic activation of Bid. This not only suggests a mechanism for anoikis regulation, but importantly highlights a physiological form of apoptosis where Bid functions in the absence of caspase 8 activation. Furthermore, our data indicates that the ability of Bid to regulate the function of pro-apoptotic Bcl-2 proteins like Bax does not require any direct interaction between them.

Acknowledgements.
The authors are grateful to Prof. Charles Streuli, Dr. Vasken Ohanian and Dr. Mauro Degli Esposti for comments on the manuscript. APG is a Wellcome Trust Research Career Development Fellow.
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**Figure legends.**

Fig. 1. Full-length Bid translocates to the membrane fraction of epithelial cells during anoikis.

a) FSK-7 cells in monolayer culture were either left attached (Adh) or detached and maintained on poly-HEMA for 15 min, 1 hr or 4 hr. Cells were separated into soluble (sol) and membrane (mem) fractions. Samples were separated by SDS-PAGE and immunoblotted for Bid, APAF-1, mitochondrial Hsp70 (mtHsp70), or active caspase 3.

b) Fractions as in a) were immunoblotted for Bax, Bcl-XL, Bak, and Bad.

c) Samples prepared as in a) were immunoblotted for Bid. Samples were run along side 1ng of recombinant tBid. tBid was only just detectable in the membrane fraction of detached cells after prolonged exposure of the immunoblot (lower panel).

Fig. 2. Bid translocates to mitochondria during anoikis.

a) Cell lysates from cells transiently expressing GFP, GFP-Bid, or Bid-GFP were immunoblotted with an anti-GFP antibody.

b) Cells transiently expressing GFP, GFP-Bid or Bid-GFP, along with the mitochondrial marker HcR-XT, were either left adherent, or detached and maintained on poly-HEMA for 1 hour before cytopinning onto glass slides. GFP and HcRed subcellular localisation was determined by fluorescent microscopy. Both GFP-Bid and Bid-GFP colocalised with HcR-XT in detached cells, but not in adherent cells where it remained cytosolic. Scale bars are 10µm.

Fig. 3. Bid translocation to mitochondria does not require caspase activity.

a) FSK-7 cells were pre-treated either with DMSO (no inhibitor), 100µM zVAD-fmk, or 100µM IETD. Cells were either left adherent or detached for 15 minutes before separating into soluble (s) and membrane (m) fractions. Fractions were separated by SDS-PAGE and immunoblotted for Bid and actin.

b) FSK-7 cells were transiently transfected with pCDNA6 (mock), pCDNA6-WT-Bid-V5 (WT), or pCDNA6-D59E-Bid-V5 (D59E). Cell lysates were prepared and expressed Bid
immunoprecipitated using a monoclonal anti-V5 antibody. Whole cell lysates (WCL) and anti-V5 immunoprecipitates (IP:V5) were separated by SDS-PAGE and immunoblotted for Bid (top panel). Both WT- and D59E-Bid-V5 migrated slightly slower than endogenous Bid (indicated by arrows). V5 immunoprecipitates were incubated with recombinant caspase 8, separated by SDS-PAGE and immunoblotted for Bid (lower panel). Only WT-Bid-V5 produced a lower molecular weight band indicative of tBid.

c) Cells expressing either WT-BidV5 or D59E-BidV5 were left adherent (Adh) or detached (poly-HEMA) for various times. Soluble (sol) and membrane (mem) fractions were prepared, separated by SDS-PAGE and immunoblotted for Bid and actin.

d) Adherent or detached (1 hour on poly-HEMA) cells expressing WT- or D59E-BidV5, along with the mitochondrial marker YFP-XT. The subcellular localisation of BidV5 was determined by immunostaining fixed cells with an anti-V5 monoclonal antibody followed by a Cy3 conjugated anti-mouse secondary. In detached cells, both WT and non-cleavable Bid V5 colocalised with YFP-XT. Both forms of Bid showed a diffuse localisation in adherent cells. Scale bars are 10µm.

Fig. 4. Bid has a role in anoikis regulation.

a) FSK-7 cells were transiently transfected with pCDNA6 (mock), or with pCDNA6 expressing WT or D59E-Bid-V5. Cells were either left adherent or detached for 5 hours. Transfected cells were identified by immunostaining with an anti-V5 monoclonal antibody and an anti-mouse Cy3 secondary. Apoptosis in V5 positive cells was quantified by nuclear morphology following staining with Hoescht. Results are from three independent experiments, error bars representing standard error of the mean.

b) FSK-7 cells were transfected with control pSilencer, pSilencer targeted to murine Bid, or empty pSilencer1.0. Cells were co-transfected with pEGFP. GFP positive cells were collected by FACS 48 hours post transfection. Whole cell lysates were separated by SDS-PAGE and immunoblotted for Bid and mtHsp70.

c) Cell were transfected with control pSilencer or Bid pSilencer. 48 hours post transfection, cells were either left adherent or detached and plated onto poly-HEMA for 5 hours. Cells were stained with Hoescht and apoptosis quantified in GFP positive cells.
on the basis of nuclear morphology. Results are from three independent experiments, error bars representing standard error of the mean.

Figure 5. Bid translocates independently of Bax during anoikis.
a) Cells were transfected with control pCDNA6, or pCDNA6 expressing WT-BidV5, D59E-BidV5, or Bax. All were co-transfected with either pEGFP or pEGFP-Bcl-XL. Cells were detached for 5 hours. Apoptosis was quantified in transfected cells following Hoescht staining by nuclear morphology. Results are from three independent experiments, error bars representing standard error of the mean.

b) Cells were transfected with GFP-Bax or Bid-GFP, with or without CFP-Bcl-XL. Subcellular localisation of CFP and GFP was determined in fixed cells by confocal microscopy. Coexpression of CFP-Bcl-XL inhibits translocation of Bax, but not Bid, following detachment. Scale bars are 10µm.

Fig. 6. Bid translocation and anoikis induction do not require interactions with other Bcl-2 family proteins.
a) Adherent or detached cells (poly-HEMA) were separated into soluble (sol) or membrane fractions. The membrane fraction was then extracted in CHAPS. The CHAPS insoluble material (insol) was solubilised in SDS-PAGE sample buffer. Equal amounts of each was separated by SDS-PAGE. Fractions were immunoblotted for Bid and Bax. As we have previously shown, following 4 hours detachment, the majority of Bax is in the CHAPS insoluble fraction. Conversely, all the membrane associated Bid is extracted by CHAPS.

b) Membrane fractions from adherent cells, or cells detached for 15 minutes or 1 hour, were separated by S100 gel filtration. Every third fraction collected was separated by SDS-PAGE and immunoblotted for Bid, Bax, Bcl-XL and Bak. Bid is always isolated as a monomer. Other Bcl-2 family proteins are always associated with high molecular weight complexes. Only the 15 minute samples are shown for Bad and Bak. No difference was seen for those molecules at any time point. Approximate sizes were determined by running molecular weight standards.
c) Cell lysates were immediately crosslinked with BS3 following Dounce homogenisation, prior to sub-cellular fractionation into soluble (sol) and membrane (mem) fractions. These were then separated by SDS-PAGE and immunoblotted for Bid. Membrane associated Bid is indistinguishable from cytosolic Bid. Note that following crosslinking there is a slight alteration in the mobility of Bid.

d) Schematic diagram of BidV5 mutants generated. Cleavage sites recognised by caspase 8 and granzyme b are indicated.

e) BH3 domain mutants that abolish binding to Bax and Bcl-2 do not inhibit sensitisation to anoikis. Cells transiently expressing BidV5 mutants were either left adherent or detached for 5 hours. Cells were immunostained for V5 and apoptosis assessed in V5 positive cells by Hoeschst staining and nuclear morphology. p15Bid was constitutively apoptotic in adherent cells. Both the mIII3 and mIII4 mutants sensitised cells to anoikis in an identical manner to WT-BidV5.

f) Bid translocation is not dependent on the BH3 domain. Cells expressing WT-BidV5, p15BidV5 or p11BidV5, along with YFP-XT, were immunostained with an anti-V5 monoclonal antibody. Both p11BidV5 and p15BidV5 constitutively localised to mitochondria. p15BidV5 expressing cells were grown in the presence of 100µM zVADfmk. WTBidV5 and p11BidV5 did not induce apoptosis and caspase inhibitors were not required. Scale bars are 10µm.

g) Translocation of FL-Bid to the membrane fraction does not require interactions with Bcl-2 or Bax. Cells expressing WTBidV5, mIII3BidV5 or mIII4BidV5 were left adherent or detached for 1 hour. They were then separated into soluble (s) or membrane (m) fractions. Fractions were immunoblotted for Bid and mtHsp70. All three forms of Bid translocated to the membrane fraction in detached cells.

Fig. 7. FL-Bid translocation to mitochondria is regulated in part by PI3-kinase. FSK-7 cells were transiently transfected with either WT- or D59E-BidV5, along with pCDNA3, pCDNA3.DN-p85 or pCDNA3.DNFAK, and pEYFP-XT. Cells were fixed 18 hr post transfection and immunostained for V5. Scale bar 10µm.
Valentijn and Gilmore Fig. 1a,b
Valentijn and Gilmore Fig. 1c
Valentijn and Gilmore Fig.2a
Valentijn and Gilmore Fig. 2b
Valentijn and Gilmore

Fig. 3a, b.
Valentijn and Gilmore
Fig. 3c, d.
Valentijn and Gilmore Fig. 4a, b, c.
Valentijn and Gilmore Fig. 5a,b

(a) Bar graph showing the percentage of apoptosis for different constructs.

(b) Confocal images of adherent and detached cells expressing GFP-Bax, CFP-Bcl-XL, GFP-Bid, and CFP-Bcl-XL.
Valentijn and Gilmore Fig.6a,b,c
Valentijn and Gilmore Fig. 6d,e.
Valentijn and Gilmore Fig. 6f,g.
Valentijn and Gilmore Fig. 7
