Bim Is an Apoptosis Sensor That Responds to Loss of Survival Signals Delivered by Epidermal Growth Factor but Not Those Provided by Integrins*

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Anoikis is a rapid apoptosis response that is initiated within a few minutes after inhibition of integrin signaling. In mammary epithelia, anoikis is mediated by subcellular translocation of Bax from the cytosol to mitochondria where it activates the intrinsic apoptosis pathway. The Bcl-2 homology 3 domain-only protein, Bim, has been proposed to have a key role in the apoptosis response of an epithelial cell line with reduced sensitivity to loss of integrin signaling, which undergoes anoikis over a period of several days in suspension culture. Here we tested the involvement of Bim in the rapid anoikis response of mouse mammary epithelial cells and discovered that Bim does not have a role in detecting integrin-mediated signals. Instead Bim senses the loss of survival cues mediated by epidermal growth factor. Cell lines selected over many passages in culture have lost much of their sensitivity to anoikis signals arising from an altered cellular microenvironment and may undergo apoptosis through acquired mechanisms.

The maintenance of cellular homeostasis is fundamental for tissue integrity in multicellular organisms. Apoptosis is a highly conserved mechanism that has evolved to maintain cell numbers and cellular positioning within tissues comprised of different cell compartments. Competition by survival signals from soluble ligands, ECM1 (extracellular matrix) ligands, and adjacent cells provides the major driving force for maintaining normal tissue architecture in developed animals. Different cell compartments. Competition by survival signals from soluble ligands, ECM1 (extracellular matrix) ligands, and adjacent cells provides the major driving force for maintaining normal tissue architecture in developed animals. Different ECM environments contribute differential thresholds for survival, thereby orchestrating the maintenance of cell positioning (1); however, the mechanisms linking ECM signals to the intracellular apoptosis machinery have not been fully elucidated. It is likely that they have evolved divergently in different cell types, since primary fibroblasts, endothelial cells, and epithelia utilize different integrins and different signaling pathways to remain alive (2–7).

One strategy to investigate the mechanisms of adhesion-dependent survival involves the use of culture models where integrin-mediated signaling is completely abrogated. Since this approach allows early events in ECM survival signaling to be synchronized, it permits dissection of the biochemical pathways involved. The apoptosis that results within this experimental paradigm is extremely rapid and is termed anoikis. It has emerged that in epithelia isolated from mammary gland, integrin-mediated signaling maintains survival by regulating the intrinsic mitochondrial pathway for apoptosis (1, 6, 8, 9).

Integrins are heterodimeric transmembrane receptors that assemble multiprotein structural and signaling complexes at sites of cell-ECM adhesion. In their ligand-binding conformation, integrins activate specific enzymes associated with adhesion complexes, and one of these, FAK (pp125 focal adhesion kinase), links adhesion survival ligands in mammary epithelia to the pro-apoptotic protein Bax. These signals maintain Bax within the cytosol, while in their absence Bax translocates to mitochondria within 15 min of removing integrin signals, where it leads to the export of cytochrome c and caspase activation (6, 8, 9). Bax activation may require the involvement of a class of Bcl-2 family proteins that are homologous only within their Bcl-2 homology 3 domain (the BH3-only proteins). Several BH3-only proteins are expressed in mammary epithelia; Bad relays death decisions to mitochondria in cells lacking sufficient GFs (growth factors) (10, 11), while Bim has recently been suggested to mediate epithelial anoikis (12, 13).

Bim is a potent pro-apoptotic protein that functions in the intrinsic pathway and is involved with apoptosis induced through either the withdrawal or inhibition of GF signaling (14–17) or the exposure of cells to pro-apoptotic triggers (18–21). The apoptotic activity of Bim is kept under control through several mechanisms. In healthy cells, Bim is phosphorylated on Ser/Thr residues by an Erk-dependent pathway that either directly inhibits its pro-apoptotic function (14) or targets it for ubiquitin-mediated proteolysis (22). Some types of healthy cells may also sequester Bim on dynemin, thereby excluding it from mitochondria (23). In response to the loss of GF signaling, activation of Foxo transcription factors leads to elevated Bim expression (15, 16), which occurs coincidently with its dephosphorylation and activation.

Increased Bim levels have been detected after loss of cell adhesion in some epithelial cell lines, and it has been argued that this is responsible for anoikis (13). However many cell types have been selected to grow in culture over a long time span, and their requirement for adhesion to survive has become considerably diminished; such cells die over a period of several days rather than in a matter of hours. It is of concern that cells...
that are not strictly adhesion-dependent are used to delineate mechanisms for adhesion-mediated survival signaling. In those where integrins contribute significantly to GF signaling (24, 25), any pro-apoptotic effects of Bim could be interpreted on the basis of reduced GF responsiveness after removing adhesion signals, rather than altered integrin signals per se. The aim of the study reported here was to resolve whether Bim is an anoikis sensor that responds directly to the loss integrin-signaling, cells cultured in growth medium containing 2% fetal calf serum, 5 ng/ml EGF, and 5 μg/ml insulin were detached from the substrate with 5 mg/ml trypsin (Sigma), immediately washed in GF-free medium, and plated in growth medium or GF-free medium onto dishes coated with polyhydroxyethylmethacrylate (poly-HEMA; Sigma) for various times. For inhibitor studies, cells were preincubated 1 h prior to the start of the experiment and throughout culture with: 50 μM cycloheximide (Calbiochem catalog number 259764); 10 μM LY294002 (Calbiochem catalog number 441172); 10 μM U0126 (Calbiochem catalog number 92626); 5 μM U0124 (Calbiochem catalog number 662006); 5 μM ZD1839 (AstraZeneca). MCF-10A cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 5% horse serum, 10 μg/ml insulin, 1 ng/ml cholera toxin, 100 μg/ml hydrocortisone, with 5% CO₂.

**Gene Silencing with Small Interference RNA (siRNA)—siRNA sequences were selected for Bim gene silencing that correspond to the coding region 43–64 relative to the first nucleotide of the start codon of the published mouse Bim-EL cDNA. They do not match any other sequence in the GenBank data base. Bim siRNA DNA was cloned into the siRNA expression vector pSilencer3.1-H1 hygro (Ambion). Sense strand siRNA (5′-GCGGCAUCAGGACGCUCAUCCGUUAGTT-3′) and antisense strand siRNA (5′-CAGGCGUCAUGCCGACCGCUUAGTT-3′) were used. FSK-7 cells were cultured on 60-mm dishes until 75% confluent and transfected with 3 μg of either the pSilencer-Bim-siRNA sequence or vector DNA and 1 μg of pEGFP plasmid DNA (enhanced GFP under the control of a cytomegalovirus promoter). 48 h after transfection, cells were sorted by using a FACS Vantage flow cytometer on the basis of GFP fluorescence. Cells were re-cultured after sorting and then subjected to anoikis and growth factor-withdrawal experiments.

**RESULTS**

**Regulation of Bim Phosphorylation and Expression Do Not Correlate with Detachment-induced Anoikis**—Several isoforms of Bim have been detected in a variety of cell types. To determine which ones are present in primary mouse mammary epithelial cells, immunoblots of cell extracts were probed with an antibody raised against amino acids 22–40 of human (and mouse) Bim-EL, Bim-L, and Bim-S (Calbiochem catalog number 202000), which specifically recognizes recombinant Bim in immunoblots (Fig. 1A). Multiple bands migrating around 25 kDa, which represent Bim-EL, are present in both adherent and detached cells (Fig. 1B and C, lanes 1). The upper band corresponds to hyperphosphorylated Bim (17, 22, 27) and largely disappears after GF withdrawal (the hyperphosphorylated form of Bim is indicated by an asterisk throughout the manuscript; also see Fig. 1D and compare lanes 1 and 2). Bim-L and Bim-S are not detected in mammary cells, even though this antibody successfully recognizes all three isoforms in other cell types (data not shown).

In adherent cells, Bim is largely phosphorylated in the presence of GF (Fig. 1B, lanes 5–8). Following GF withdrawal, Bim and Erk become dephosphorylated within 1 h, and subsequently the levels of Bim rise (Fig. 1, B, lanes 1–4, and D, lanes 1 and 2). Caspase 3 activation begins at 4 h. When cells are detached from the ECM, the GF response of Bim is similar to that in adhered cells; Bim and Erk remain phosphorylated in the presence of GF (Fig. 1C, lanes 5–8, D, lanes 3 and 4, and Fig. 1E), while removing GF results in Bim and Erk de-phosphorylation followed by increased Bim levels (Fig. 1C, lanes 1–4, and D, lane 5). Thus, Bim phosphorylation levels are regulated by GFs but not by ECM. Although detachment has no effect on Bim, it has a dramatic effect on apoptosis. In the absence of GFs, caspase 3 activation occurs at 4 h in both adherent and detached cells, but the intensity of caspase activation is considerably higher in the latter (Fig. 1, B and C, lanes 3 and 4). However, in the presence of GFs, caspase 3 is only activated after cell detachment (Fig. 1, B and C, lanes 7 and 8).

Increased levels of Bim following GF withdrawal in some other cell types are due to de novo protein synthesis, because the removal of interleukin-3 from Ba/F3 cells or NGF from sympathetic neurons activates Foxo-dependent Bim transcription (15, 16). We have shown previously that protein synthesis is not required for anoikis (9). To further break the connection between increased levels of Bim and anoikis in mammary epithelia, we examined detached cells in which protein synthesis had been blocked with cycloheximide (CHX). In the absence of GF, apoptosis (Fig. 1F) and the appearance of active caspase 3 (Fig. 1G, lanes 7–9) begin 4 h after detachment, increasing thereafter. Bim levels rise over this time course. CHX prevents Bim elevation (Fig. 1G, lanes 10–12), yet caspase 3 is cleaved with identical kinetics. Moreover apoptosis occurs similarly in detached cells treated with GF, either in the presence or absence of CHX, and Bim remains phosphorylated (Fig. 1, F and G, lanes 1–6). Thus, anoikis does not depend on an increase in the levels of Bim. In contrast to some cell types, primary mammary epithelial cells still respond to GFs after they have been in suspension for several hours, retaining Erk phosphorylation over this time frame (Fig. 1C). Nevertheless, these detached cells show the same rate of caspase 3 cleavage as those cultured in the absence of GFs, indicating that the Erk-Bim pathway does not contribute to anoikis regulation. Primary cell cultures are particularly sensitive to the induction of apoptosis both by withdrawal of soluble survival factors and ECM, and we wished to determine whether some of the mammary epithelial cell lines used as models for anoikis show the same lack of requirement for Bim. In particular we wished to compare FSK-7 and MCF-10A cells.

FSK-7 is a mouse mammary epithelial cell line that we have used previously in studies to examine anoikis mechanisms, because it behaves in a similar manner to primary cells (6, 8, 9). In detached cells cultured in the absence of GFs, Bim is dephosphorylated throughout, and caspase 3 becomes cleaved after 4 h (Fig. 1H, lanes 5 and 6). This is similar to the kinetics of apoptosis in primary cells (Fig. 1C, lanes 3 and 4). In the absence of GFs, Erk and Bim remain phosphorylated, indicating that integrins are not necessary for GF signaling (Fig. 1H, lanes 7–12). Apoptosis occurs in these cells with similar kinetics to that in the presence of GFs (i.e. it begins at 4 h; Fig 1H, lanes 11 and 12, and Fig. 1I), but Bim is still phosphorylated, and there is no visible increase in Bim level. This suggests that
FIG. 1. **Loss of integrin-mediated adhesion leads to anoikis independently of Bim.** In all the Figures in this paper, the data presented are representative of at least three independent experiments. All Bim blots were probed with the Calbiochem antibody unless otherwise indicated. The *asterisk* donates phosphorylated Bim. A, FSK-7 cells were transfected with eGFP, eGFP-Bax, or eGFP-Bim-EL, and after 48 h, cells were lysed and assayed by immunoblotting for GFP (upper panel). eGFP-Bax and eGFP-Bim-EL migrate at a molecular weight consistent with their predicted masses. Blots were stripped and reprobed for Bax or Bim (lower panels). B, primary mouse mammary epithelial cells cultured normally with GF (2% fetal calf serum, 5 ng/ml EGF, and 5 μg/ml insulin) were washed and then incubated in the presence or absence of GF for indicated times, and...
Bim is not involved with anoikis regulation in FSK-7 mammary cells. Slightly more apoptosis occurs in detached FSK-7 cells that are also deprived of serum for 8 h (Fig. 1H, compare lanes 6 and 12, and Fig. 1F), indicating that in this cell line growth factors and adhesion both contribute to survival, while in primary epithelial cells adhesion is dominant.

MCF-10A is a non-transformed human breast epithelial line that has been used to study the mechanism of adhesion-mediated apoptosis (12, 13). In suspended cells cultured without GFs, Bim and Erk are dephosphorylated, but caspase 3 cleavage is only revealed after considerable times (i.e., after 16 h; Fig. 1J, lanes 1–6). In the presence of GFs, Erk and Bim are phosphorylated for 4 h, but interestingly in this cell line they cannot maintain the Erk-Bim axis at later time points suggesting that integrins are necessary for long term GF signaling (Fig. 1J, lanes 7–12). We also observed that another antibody to Bim (Stressgen catalog number AAP-330) does not recognize phosphorylated Bim, and thus, the increased “levels” of Bim during anoikis described by others (13) is more likely to represent dephosphorylation concomitant with loss of Erk signaling. Even though Bim becomes dephosphorylated in the presence of GF, caspase 3 activation in the bulk cell population is not apparent (Fig. 1J, lanes 7–12), while examining the nuclei in suspended single cells reveals that less than 20% has undergone apoptosis at 24 h, and many cells are still alive after 48 h (Fig. 1K). This contrasts with both primary (Fig. 1F) and FSK-7 cells (6, 9), which are largely all dead after 24 h in suspension. We interpret these results to indicate that MCF-10A cells are not truly adhesion-dependent for survival. They lack the sensitivity to respond to lost integrin signals and eventually undergo apoptosis through an alternative mechanism.

Together, our results demonstrate that changes in Bim levels and phosphorylation do not occur in response to loss of integrin signaling during the rapid anoikis of adhesion-dependent cells but rather are caused by the withdrawal of GFs.

Bim Is Required for Apoptosis following EGF Withdrawal but Not Anoikis—To investigate the requirement for Bim in anoikis and EGF-regulated apoptosis, gene silencing experiments were performed by transfection with plasmids encoding siRNA duplexes specific for mouse Bim, and cells were then either detached from the matrix or EGF was withdrawn. In these experiments cells were co-transfected with a GFP-expressing plasmid, and FACS was used to collect the transfected cells before examining Bim levels and performing apoptosis experiments.

48 h after transfection with siRNA, the level of endogenous Bim-EL in FSK-7 cells becomes reduced to less than 15% of that in control cells (Fig. 2A). The transfected cells were then either kept in suspension in whole growth medium or were replated and cultured in the presence or absence of EGF or treated with the EGFR antagonist ZD1839 (28). As expected, the control-transfected cells undergo apoptosis when they are kept in suspension for 24 h, due to the loss of integrin signaling (Fig. 2B). However, the specific down-regulation of Bim-EL by siRNA has no effect on the extent of anoikis. Moreover, the ratio of phosphorylated to unphosphorylated Bim in the small amount of Bim remaining after RNA interference treatment is no different to that in control cells (ratio as determined by scanning densitometry is 0.92 in the control and 0.88 in the siRNA-treated sample). In contrast, the apoptosis in adherent cells that results from either EGF withdrawal or inhibition of EGF signaling by ZD1839 is reduced by ~50% in cells with reduced Bim-EL expression (Fig. 2C).

Together these data confirm that Bim is not required for anoikis. Instead they demonstrate that it is a key mediator of the apoptosis response resulting from EGF withdrawal.

EGF Regulates Bim through a MEK Pathway in Mammary Epithelial Cells—To confirm the critical and direct role for GFs in Bim phosphorylation, adherent mammary cells were treated with EGF in the absence and presence of various pharmacological inhibitors of signaling. Cells cultured without GF for 3 h do not have detectable Bim phosphorylation (as revealed by its slower migrating form in polyacrylamide gels), but this is induced within 15 min of EGF addition, simultaneously with Erk phosphorylation (Fig. 3A). EGF-mediated Bim and Erk phos-
phosphorylation is completely abrogated by ZD1839 as well as U0126, a MEK inhibitor (29) (Fig. 3B). Bim can therefore be phosphorylated downstream of EGFR via a MEK pathway.

EGF withdrawal leads to Bim dephosphorylation and a subsequent increase in its expression levels (Fig. 3C). An increase in Bim level in primary sympathetic neurons and pro-B cells after removing GFs is due to Foxo-mediated transcription (15, 16). However, if the elevation of Bim in mammary epithelia is blocked with CHX, caspase 3 cleavage still occurs (Fig. 3C). Thus, when EGF is removed from adherent mammary cells under normal growth conditions, the dephosphorylation of Bim is sufficient for apoptosis.

Some studies have revealed a role for PI3K and PKB in Bim phosphorylation (15, 18). However, Bim remains phosphorylated in the presence of LY294002 when PKB is not activated, and cells containing phospho-PKB in the presence of U0126 do not have phospho-Bim (Fig. 3B). Moreover the IGF-PKB axis does not induce Bim phosphorylation (Fig. 3D). Thus, the PKB pathway may not play a significant role in regulating Bim in mammary cells.

These results concur with those of others who have demonstrated a role for Erk in Bim phosphorylation (13, 14, 22, 30, 31), and they extend them by showing that an EGF-MEK-Bim axis is active in mammary epithelia.

**DISCUSSION**

Bim is normally expressed at low levels, and its pro-apoptotic activity is kept in check by both phosphorylation (14, 27) and ubiquitination-mediated turnover (30, 32, 33). When cells respond to a reduction of external survival cues, the apoptotic potential of Bim can be realized through both increased expression and dephosphorylation. Many signaling pathways shown to regulate Bim are controlled through GF signaling. Integrin-mediated adhesion is critical for the survival of many cell types, and removing the ECM cues rapidly commits them to apoptosis. Bim has previously been highlighted as a candidate protein to detect loss of adhesion signals, but those studies (12, 13) were done in cell lines that have considerably reduced protein to detect loss of adhesion signals, but those studies (12, 13) were done in cell lines that have considerably reduced dependence on integrin survival signals. Here we provide three lines of evidence that Bim does not have this role, rather it is a sensor for reduced thresholds of GF signals.

First, the levels and phosphorylation of Bim do not change in primary mammary epithelial or FSK-7 cells after detachment from ECM in the presence of GFs, yet cells enter apoptosis (30, 32, 33). When cells respond to a reduction of external survival cues, the apoptotic potential of Bim can be realized through both increased expression and dephosphorylation. Many signaling pathways shown to regulate Bim are controlled through GF signaling. Integrin-mediated adhesion is critical for the survival of many cell types, and removing the ECM cues rapidly commits them to apoptosis. Bim has previously been highlighted as a candidate protein to detect loss of adhesion signals, but those studies (12, 13) were done in cell lines that have considerably reduced dependence on integrin survival signals. Here we provide three lines of evidence that Bim does not have this role, rather it is a sensor for reduced thresholds of GF signals. Some studies have revealed a role for PI3K and PKB in Bim phosphorylation (15, 18). However, Bim remains phosphorylated in the presence of LY294002 when PKB is not activated, and cells containing phospho-PKB in the presence of U0126 do not have phospho-Bim (Fig. 3B). Moreover the IGF-PKB axis does not induce Bim phosphorylation (Fig. 3D). Thus, the PKB pathway may not play a significant role in regulating Bim in mammary cells.

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First, the levels and phosphorylation of Bim do not change in primary mammary epithelial or FSK-7 cells after detachment from ECM in the presence of GFs, yet cells enter apoptosis within 4 h (Fig. 1, F and I). Indeed our previous data show that cells irreversibly commit to apoptosis much earlier than that, between 1 and 2 h after removing integrin signals (8). Second, any possible involvement of increased Bim levels that might have gone undetected in our experiments with detached cells is ruled out by the use of CHX. This blocks protein synthesis in mammary cells. Indeed our previous data show that cells irreversibly commit to apoptosis much earlier than that, between 1 and 2 h after removing integrin signals (8). Second, any possible involvement of increased Bim levels that might have gone undetected in our experiments with detached cells is ruled out by the use of CHX. This blocks protein synthesis in mammary cells. Indeed our previous data show that cells irreversibly commit to apoptosis much earlier than that, between 1 and 2 h after removing integrin signals (8). Second, any possible involvement of increased Bim levels that might have gone undetected in our experiments with detached cells is ruled out by the use of CHX. This blocks protein synthesis in mammary cells.

The mechanism of anoikis varies among cell types and involves diverse pathways such as JNK (c-Jun NH2-terminal kinase) and p53 in fibroblasts (2, 34), Erk and cFLIP in endothelial cells (5), and Ras, PI3K, and PKB in epithelia (4, 7). Our laboratories have provided extensive evidence that anoikis in mammary epithelial cells is regulated through the mitochondrial pathway for death and involves the rapid activation of Bax (6, 8, 9). Bax is required because microinjecting antibodies against its BH3 domain (6) or deleting the Bax gene (8) abrogates anoikis.

Bax is normally present as a monomer in the cytosol, and following the receipt of a suitable signal such as loss of adhesion, it undergoes a conformation change to reveal the carboxyl-terminal hydrophobic tail and moves to mitochondria (35).
Since Bax initially remains as a monomer once it has arrived in mitochondria (8), BH3-only proteins are unlikely to bind to Bax during the translocation event. Subsequently Bax undergoes a further conformation change and then oligomerizes, becoming involved with mitochondrial pore formation and high molecular weight complexes. In mammmary cells Bim is constitutively associated with mitochondria (rather than microtubules),2 but the data in this paper argue that Bim is not involved in any of these mitochondrial processes involving Bax during anoikis. Moreover gel filtration experiments only reveal co-localization of Bax and Bim during the late post-commitment stages of apoptosis.2 We have recently shown that Bax-dependent anoikis instead requires another member of the BH3-only family, full-length Bid (36).

Our experiments provide new information about the involvement of Bim in GF signaling. Several GFs mediate at least partial survival signaling through inhibition of the pro-apoptotic activity of Bim. These include NGF for PC12 cells and primary sympathetic neurones (14, 15), serum for CC139 fibroblasts (31), and interleukin-3 for various hematopoietic cells (16). We now extend this by demonstrating the involvement of Bim in EGF-driven survival of adherent primary mammary epithelial cells. This is consistent with work suggesting a role for Bim in the co-ordinate integrin/EGF receptor survival response of MCF-10A epithelial cells (13), but our data do not support a role for Bim downstream of integrins. GF signaling in primary epithelial cells can be maintained over several hours in the absence of integrin signaling, and these cells undergo apoptosis through a Bax-dependent mechanism.

We view anoikis to be the rapid apoptosis response of an altered cellular environment, which might, for example, represent a change in the threshold of survival signaling when cells extend adhesion processes to sample new microenvironments during migration. Many cell lines have been selected to grow efficiently in tissue culture and have become less adhesion-dependent for survival; some, such as MCF-10A, take several days to undergo apoptosis in the absence of direct integrin signals. We suggest that these are not good models to dissect the mechanisms of anoikis because their apoptosis-regulatory pathways are altered, and indeed their integrin and GF receptor signaling can become linked to one another. By working with a cell system where essential ECM and GF signals can be separated functionally, we have found distinct links between Bcl-2 family members and the intrinsic mitochondrial apoptosis pathway: ECM signals through a PI3K pathway to Bax (6), while EGF suppresses the activity of Bim.

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