

# Integrin-mediated Survival Signals Regulate the Apoptotic Function of Bax through Its Conformation and Subcellular Localization

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**Abstract.** Most normal cells require adhesion to extracellular matrix for survival, but the molecular mechanisms that link cell surface adhesion events to the intracellular apoptotic machinery are not understood. Bcl-2 family proteins regulate apoptosis induced by a variety of cellular insults through acting on internal membranes. A pro-apoptotic Bcl-2 family protein, Bax, is largely present in the cytosol of many cells, but redistributes to mitochondria after treatment with apoptosis-inducing drugs. Using mammary epithelial cells as a model for adhesion-regulated survival, we show that detachment from extracellular matrix induced a rapid translocation of Bax to mitochondria concurrent with a

conformational change resulting in the exposure of its BH3 domain. Bax translocation and BH3 epitope exposure were reversible and occurred before caspase activation and apoptosis. Pp125FAK regulated the conformation of the Bax BH3 epitope, and PI 3-kinase and pp60src prevented apoptosis induced by defective pp125FAK signaling. Our results provide a mechanistic connection between integrin-mediated adhesion and apoptosis, through the kinase-regulated subcellular distribution of Bax.

**Key words:** apoptosis • Bax • mammary • adhesion • pp125FAK

## Introduction

Most normal cells require adhesion to extracellular matrix (ECM)<sup>1</sup> for cell growth, differentiation and survival. Adhesion dependence permits cell differentiation and growth only when the cell is in its correct environment within the organism. Cells that become displaced from their appropriate locations are deleted by apoptosis, a highly regulated process by which unwanted or damaged cells are destroyed without inducing an inflammatory response (Meredith et al., 1993; Frisch and Francis, 1994). Apoptosis is tightly controlled and its deregulation leads to degenerative conditions or to the survival of abnormal cells, contributing to the development of tumors. In the mammary gland, luminal epithelial cells are dependent upon adhesion to ECM for survival (Boudreau et al., 1995; Pullan et al., 1996; Farrelly et al., 1999; Streuli and Gilmore, 1999). Primary mam-

mary epithelial cells show a specific requirement for a laminin-rich basement membrane for survival, and undergo apoptosis over several days if they are cultured on collagen I (Pullan et al., 1996). Laminin acts as a survival ligand through  $\beta 1$  integrins and cooperates with signals from insulin to suppress mammary epithelial apoptosis (Farrelly et al., 1999). However, the molecular mechanisms that link adhesion events at the cell surface to the intracellular apoptotic machinery are not understood.

Apoptosis induced by a wide variety of factors, from growth factor starvation to cytotoxic drugs, occurs as a defined sequence of events. The apoptotic stimulus or insult must initially be sensed and transduced to the apoptotic machinery, but only after commitment to apoptosis does the execution phase occur through caspase activation. Members of the Bcl-2 family appear to function at a pivotal point in the decision process where cells become irreversibly committed to die. Some members of this family, including Bcl-x, Bcl-2, Bcl-w, and Mcl-1 promote cell survival, whereas others, such as Bax, Bak, Bad, Bik, Bid, Bok, and Bim promote apoptosis (Adams and Cory, 1998). These molecules form both homo- and heterodimers, and one possible mechanism for their control on apoptosis is that the relative proportions of their interactions regulate

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<sup>1</sup>*Abbreviations used in this paper:* BH, Bcl-2 homology; BrdU, bromodeoxyuridine; DN-FAK, dominant-negative FAK; DSS, disuccinimidyl suberate; ECM, extracellular matrix; PI 3-kinase, phosphatidylinositol 3-kinase; pp125FAK, focal adhesion kinase; polyHEMA, polyhydroxyethylmethacrylate.

the balance between apoptosis and survival, much like a rheostat (Yin et al., 1994). However, other data indicates that their pro- and anti-apoptotic functions are at least partially independent of their ability to interact with each other (Cheng et al., 1996; Simonian et al., 1996; Knudson and Korsmeyer, 1997; Simonian et al., 1997; St. Clair et al., 1997; Metcalfe et al., 1999). The crystal structure of Bcl-x suggests a potential to form membrane pores, and indeed Bcl-x, Bcl-2, and Bax all form pores in artificial bilayers (Muchmore et al., 1996; Minn et al., 1997). A number of Bcl-2 family members have a predicted transmembrane sequence at their carboxyl terminus, which localizes them to internal cell membranes including mitochondria and the ER. These proteins may act at the mitochondrial membrane by directly or indirectly inducing the release of proapoptotic molecules such as caspase 9 and cytochrome c (Kluck et al., 1997; Zou et al., 1997; Narita et al., 1998; Krajewski et al., 1999; Shimizu et al., 1999). Thus, although the mechanism of commitment to apoptosis is not yet fully understood, it may involve changes in mitochondrial permeability that are regulated by Bcl-2 family proteins.

Bax has been reported to be predominantly cytosolic in healthy cells, despite having a membrane-targeting carboxyl terminus. Bax translocates to mitochondria in a number of cell types after receipt of a death signal, such as cytotoxic insult (Hsu et al., 1997; Wolter et al., 1997; Goping et al., 1998; Gross et al., 1998; McGinnis et al., 1999). Deletion of the carboxy-terminal tail prevents Bax translocation and apoptosis after a death stimulus, suggesting that the regulation of its association with mitochondrial membranes may be a key stage in transduction of an apoptotic signal (Wolter et al., 1997). Indeed, enforced dimerization of Bax results in its constitutive targeting to mitochondria, resulting in apoptosis in FL5.15 cells (Gross et al., 1998). Bcl-2 family proteins have a number of conserved sequences termed BH domains (for Bcl-2 homology domains). The BH1, BH2, BH3, and BH4 domains have critical functions in determining the pro- and anti-apoptotic properties of the various family members, and are involved in mediating interactions between them. The BH3 domain of Bax is a region of the molecule that is critically important for the apoptosis-inducing ability of the protein (Zha et al., 1996a; Cosulich et al., 1997; Wang et al., 1998). Mitochondria-targeted Bax requires a functional BH3 domain in order to induce apoptosis. At least two domains of Bax are therefore required for its pro-apoptotic activity, a region that specifies its translocation to organelle membranes including mitochondria, and its BH3 domain.

Cells adhere to the ECM through a family of heterodimeric transmembrane receptors, the integrins. Signaling pathways regulated by adhesion include tyrosine and serine/threonine kinase activation, as well as changes in cellular pH, intracellular calcium and phospholipids (Schwartz et al., 1995). The tyrosine kinase pp125FAK (focal adhesion kinase) has been implicated as a central molecule for integrin-mediated signaling. Upon integrin ligation and clustering, pp125FAK becomes phosphorylated and activated, leading to its association with other kinases and adapter molecules, including PI 3-kinase, pp60src, p130CAS, paxillin and Grb2, which in turn leads to activation of downstream pathways (Aplin et al., 1998; Schwartz and Baron, 1999). Pp125FAK is therefore poised to regulate adhesion-

dependent cell phenotypes including survival (Gilmore and Romer, 1996; Zhao et al., 1998). Indeed, inhibition of pp125FAK by injection of anti-FAK antibodies or by expression of a dominant negative FAK results in apoptosis in serum-deprived fibroblasts and cancer cell lines (Hungerford et al., 1996; Ilic et al., 1998), whereas expression of constitutively activated pp125FAK leads to the anchorage-independent survival of MDCK cells (Frisch et al., 1996). However, although pp125FAK is a candidate for regulating adhesion-mediated survival, a link between pp125FAK and apoptosis regulatory proteins has not been identified.

In this paper, we sought to define the mechanism by which adhesion to ECM regulates the apoptotic machinery. Since Bcl-2 family proteins have a central role in apoptosis induced by a wide variety of different cellular insults, we investigated the possibility that their function is controlled by adhesion-mediated signaling. We found that the apoptotic protein Bax was largely present in the cytosol of adherent mammary epithelial cells. Detachment from ECM induced a rapid, but reversible, translocation of Bax to mitochondria concurrent with a conformational change resulting in the exposure of its BH3 domain. Bax translocation and BH3 epitope exposure occurred before commitment to apoptosis. We identified a critical role for kinase cascades in adhesion-regulated survival signaling and suggest that conformation of the Bax BH3 epitope is dependent on both pp125FAK and PI 3-kinase.

## Materials and Methods

### Cell Culture and Apoptosis Assays

Primary mouse mammary cells, isolated from pregnant ICR mice (Pullan and Streuli, 1996), and FSK-7 cells (Kittrell et al., 1992), were grown in DMEM/F12 supplemented with 2% fetal calf serum, 5 ng/ml epidermal growth factor and 880 nM insulin. To assay detachment-induced apoptosis, confluent cells were trypsinized and replated in whole medium onto dishes coated with polyhydroxyethylmethacrylate (poly-HEMA). In the case of inhibitor experiments, cells were preincubated with inhibitors 1 h before trypsinization and throughout incubation on poly-HEMA. Inhibitors were obtained from Calbiochem and used at the following concentrations: cycloheximide was used at 25  $\mu$ g/ml, zVAD-fmk, and Ac-DEVD-cmk at 100  $\mu$ M, herbimycin A at 1  $\mu$ M, and wortmannin at 1  $\mu$ M; pervanadate was used at 1 mM from sodium orthovanadate and hydrogen peroxide (Sigma), and treated with catalase (Sigma) to quench unreacted hydrogen peroxide. After incubation for various times, cells were cytospun onto polylysine slides (Merck), and fixed in 2% paraformaldehyde. To quantify apoptosis, nuclear morphology was examined after staining cells with 4  $\mu$ g/ml Hoechst 33258 (Molecular Probes). To analyze DNA integrity after incubation on poly-HEMA, cells were washed in PBS and lysed in 100 mM NaCl, 10 mM EDTA, 25 mM Tris-Cl, pH 8.0, 0.65% SDS, and 500  $\mu$ g/ml proteinase K. Lysates were extracted with phenol/chloroform (1:1). DNA was ethanol precipitated, treated with RNase and separated on a 1.5% agarose gel containing ethidium bromide.

### Protein Extraction and Immunoblotting

For fractionation studies, cells were washed once in PBS before resuspending in hypotonic buffer (10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-Cl, pH 7.6, 1 mM NaFl, and 100  $\mu$ M sodium orthovanadate, containing protease inhibitors) and lysed in a Dounce homogenizer before addition of 5 $\times$  isotonic buffer (525 mM mannitol, 172 mM sucrose, 12.5 mM Tris-Cl, pH 7.6, and 2 mM EDTA). Cytosolic and membrane fractions were then centrifuged at 100,000 g. Equivalent amounts of protein were separated by SDS-PAGE and immunoblotted (Metcalfe et al., 1999). For cross-linking experiments, after incubations, cells were washed in PBS twice before incubating with 2.5 mM disuccinimidyl suberate (Pierce) in 200 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES, pH 7.5, for 30 min. The reaction was stopped by adding Tris-Cl, pH 7.6, to 20 mM. The total

lysates were boiled in SDS-PAGE sample buffer and immunoblotted with polyclonal anti-Bax 62M (Metcalfe et al., 1999), anti-calnexin (Stress Gen), and anti-active caspase 3 (a kind gift from Anu Srinivasan, Idun Pharmaceuticals, Inc., La Jolla, CA), and monoclonal rat anti-Bax (PharMingen), mouse anti-Bcl-x (Transduction laboratories), and mouse anti-COX I (Molecular Probes).

### Microinjection

GST fusion proteins were expressed in *E. coli* and purified on glutathione-agarose (Sigma) as previously described (Gilmore and Romer, 1996). FSK-7 cells were grown to confluence on coverslips before microinjecting with either GST alone or with the GST-tagged dominant negative pp125FAK

(DN-FAK) fusion protein at 3 mg/ml in 75 mM KCl, and 10 mM potassium phosphate, pH 7.5. Cells were fixed in 2% paraformaldehyde either 1 or 5 h postinjection before immunostaining.

### Transient Transfections

The plasmid pSG5.p110CAAX was a generous gift of Dr. Julian Downward (ICRF, London, UK). TS-pp60src was kindly given by Dr. Ged Brady (University of Manchester, Manchester, UK). Both were subcloned into the expression vector pCDNA.3 to produce pCDNA.3/p110CAAX and pCDNA.3/src. pCMV3RΔp85 (referred to in text as p85ΔSH2) was kindly provided by Dr. Phill Hawkins (Babraham Institute, Cambridge, UK). Full-length murine Bax was cloned by PCR using *pfu* DNA polymerase (Stratagene) from RNA isolated from adult mouse mammary gland using PCR primers directed against the 5' and 3' ends of the coding sequence. HA-tagged Bax and Bax truncated at its carboxyl terminus at residue 172 (BaxΔCT) were generated by PCR using the 5' primer ATGTACCCATACGACGTCCAGACTACGCCATGGACGGGTCC, incorporating the HA epitope tag, TCAGCCCATCTTCTTCAGAT was used as the 3' primer for Bax, and TCACTGCCATGTGGGGGTCC for BaxΔCT. Both were cloned into pCR-script SK+ (Stratagene) and confirmed by double stranded sequencing, before subcloning into pCDNA.3 to produce pCDNA.3/HA-Bax and pCDNA.3/HA-BaxΔCT. GST-tagged DN-FAK (amino acids 839-1052) was amplified by PCR using the 5' primer GCCGCCATGTCCCTATACTA, and the 3' primer TCAGTGTGGC-CGTGTCTG, and cloned into pCDNA.3.

FSK-7 cells plated onto coverslips at  $1 \times 10^5$  cells/cm<sup>2</sup> were grown to 80–90% confluence before transfecting using lipofectamine plus (GIBCO BRL). Cells were transfected with a total of 3 μg DNA. For cotransfections, 2 μg of pCDNA.3/DN-FAK was used with 1 μg of pCDNA.3, pCDNA.3/p110CAAX or pCDNA.3/src. Cells were transfected for 3 h followed by 18 h incubation in growth medium. Detached cells were collected and cytospun onto polysine-coated slides. Both the adherent and the detached cells were immunostained. DN-FAK- or p85ΔSH2-expressing cells with apoptotic morphology were counted.

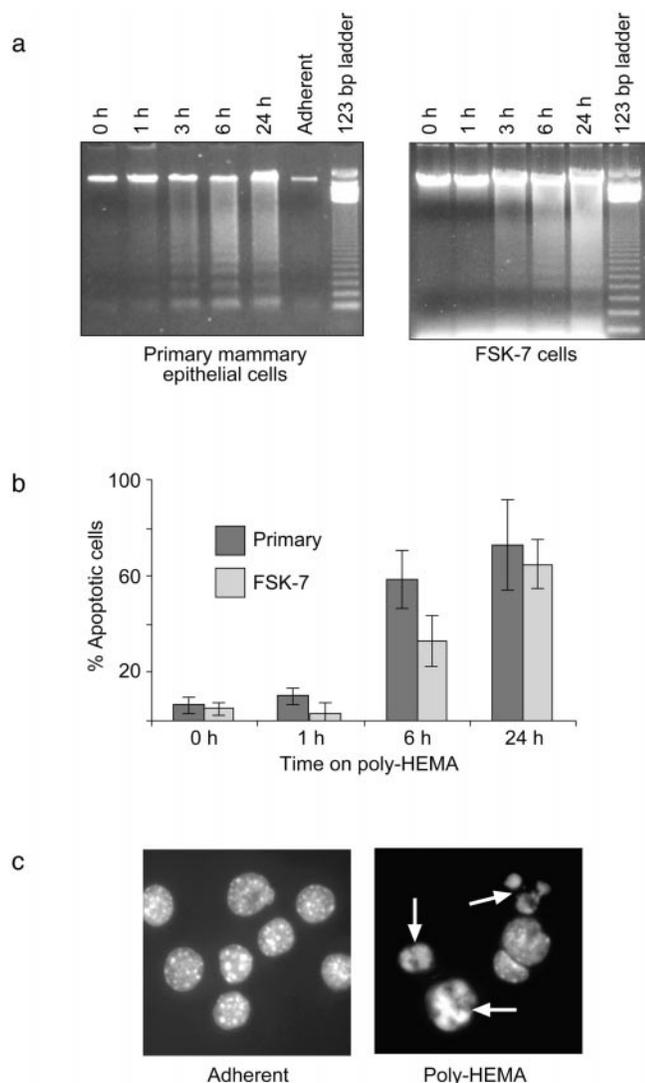
### Immunofluorescence

Cells were fixed in 2% paraformaldehyde in PBS and permeabilized in 0.5% Triton X-100. Cells were stained with anti-Bax 62M, anti-GST (Pharmacia) or the p85 subunit of PI 3-kinase (Upstate Biotechnology Inc.) in PBS with 0.1% horse serum, followed by either Cy2- or Cy3-conjugated secondary antibodies (Jackson Laboratories). Cells were counterstained with 4 μg/ml Hoechst 33258. Cells were viewed on a Zeiss Axiophot photomicroscope equipped with epifluorescence and images were taken on T-MAX 400 film. For comparison of Bax staining, all exposures and subsequent image manipulations were identical. For visualization of mitochondria, cells were incubated for 15 min before fixation with 500 nM Mitotracker green-fm (Molecular Probes).

### Results

#### Detachment-induced Apoptosis in Mammary Epithelial Cells Is Preceded by Redistribution of Bax from a Cytosolic to an Insoluble Fraction

Mammary epithelial cells require integrin-mediated adhesion to ECM for survival (Streuli and Gilmore, 1999). Primary mammary epithelial cells have been shown to undergo apoptosis when plated onto an inappropriate ECM (Pullan et al., 1996; Farrelly et al., 1999). This dependence on ECM was confirmed by the rapid onset of apoptosis when mammary cells were detached from their substrata and maintained in suspension by plating onto nonadhesive poly-HEMA. After detachment, nucleosomal DNA ladders were detectable after 3–5 h, along with a loss of cell number and increase in the proportion of cells showing morphological changes associated with apoptosis (Fig. 1). Detachment therefore served to synchronize apoptosis in ECM-dependent mammary epithelial cells. We examined a number of mammary cell lines and found that these also



**Figure 1.** Cultured primary mammary epithelial cells and mammary cell lines undergo apoptosis after detachment from ECM. (a) Confluent primary mouse mammary epithelial cells and FSK-7 cells were detached by trypsinization and maintained on poly-HEMA coated dishes for the indicated times before low molecular mass DNA was extracted and analyzed on 1.2% agarose gels stained with ethidium bromide. (b and c) Cells were incubated in the absence of adhesion on poly-HEMA coated dishes for the indicated times before being cytospun onto glass slides. The number of apoptotic cells was determined by Hoechst 33258 staining and counting of cells with typically apoptotic nuclear morphology, indicated by the arrows in c. Error bars represent SEM.

showed a strong dependence upon ECM for survival. The mouse mammary cell line, FSK-7, showed rapid apoptotic laddering when maintained on poly-HEMA (Fig. 1 a), with a time course similar to that observed for primary cells. This occurred with a loss of 60% in cell number by 24 h, and an increase in the number of cells showing the condensed and fragmented nuclei indicative of apoptosis (Fig. 1, b and c).

We wished to investigate the possible role of members of the Bcl-2 family in the ECM regulation of mammary cell survival. FSK-7 cells express a number of the known Bcl-2 family proteins, including Bax and Bcl-x. The major pro-apoptotic member of this family in FSK-7 cells, Bax, has previously been shown to display a cytosolic distribution in a number of cell types, but redistributes to mitochondria when cells are treated with apoptosis-inducing drugs (Hsu et al., 1997).

To determine if redistribution of endogenous Bax was involved in apoptosis of mammary cells detached from ECM, we compared Bax localization in FSK-7 cells in monolayer culture and after culture on poly-HEMA. Cells were lysed by swelling in a hypotonic buffer, followed by Dounce homogenization. The efficacy of homogenization was checked by trypan blue staining, and found to be >95%. The soluble and insoluble fractions were separated by centrifugation and the relative distribution of Bax was analyzed by immunoblotting (Fig. 2 a). In healthy monolayer culture, where apoptotic cells represented <4% of the population, Bax was predominantly found in the cytosol. However, in cells maintained on poly-HEMA for 1 h, Bax showed a marked redistribution to the insoluble fraction. The mitochondrial marker cytochrome oxidase I indicated that equivalent amounts of the insoluble fractions were loaded, also confirmed using the ER marker protein calnexin. The lack of these proteins in the cytosol indicated that the presence of Bax in the cytosolic compartment was

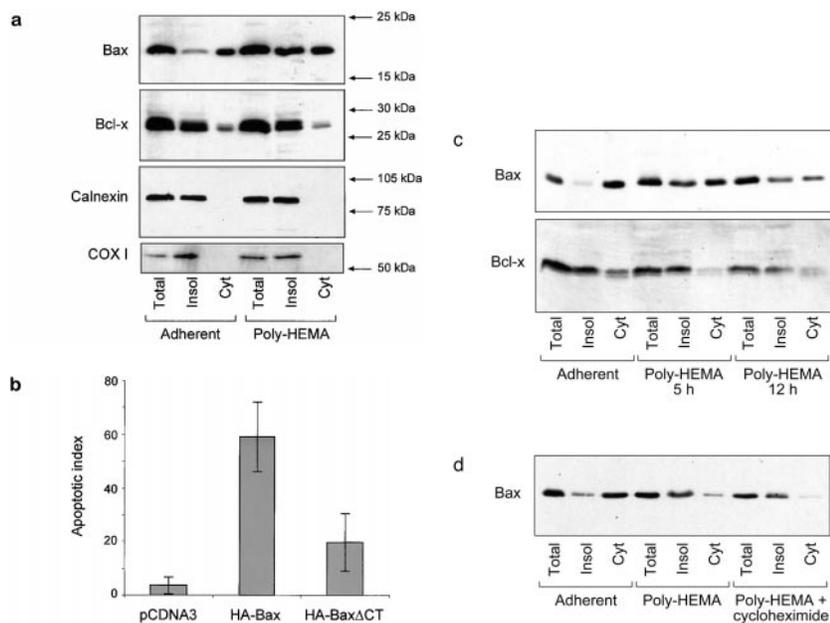
not due to organelle disruption. We also examined the major pro-survival member of the Bcl-2 family expressed in FSK-7 cells, Bcl-x. This was primarily associated with the insoluble fraction, both in adherent cells and those in suspension. Thus, loss of adhesion to ECM results in a rapid relocalization in the subcellular distribution of Bax.

Translocation of Bax to internal cell membranes, mediated by its carboxy-terminal domain, has previously been shown to be required for Bax apoptotic function in COS-7 cells (Wolter et al., 1997). We confirmed that this was also the case in mammary epithelia by transfecting FSK-7 cells with HA-tagged Bax with or without the hydrophobic carboxyl terminus (HA-Bax and HA-Bax $\Delta$ CT). Transfected cells were examined for apoptotic morphology (Fig. 2 b). We found that overexpression of HA-Bax induced three times more apoptosis than HA-Bax $\Delta$ CT. These results indicate that in FSK-7 cells, membrane targeting of Bax is required for it to induce apoptosis.

The redistribution of Bax occurred within 1 h after detachment from ECM, well before the appearance of apoptotic markers such as nuclear condensation. Bax remained associated with the insoluble fraction of cells throughout prolonged culture in suspension, while cells were undergoing apoptosis (Fig. 2 c). Redistribution of Bax was not associated with any change in the total amount of the protein detected, and was, therefore, unlikely to require the de novo synthesis of Bax. This was confirmed by treating cells for 1 h before detachment from ECM with cycloheximide, which had no effect on its redistribution to the insoluble fraction (Fig. 2 d).

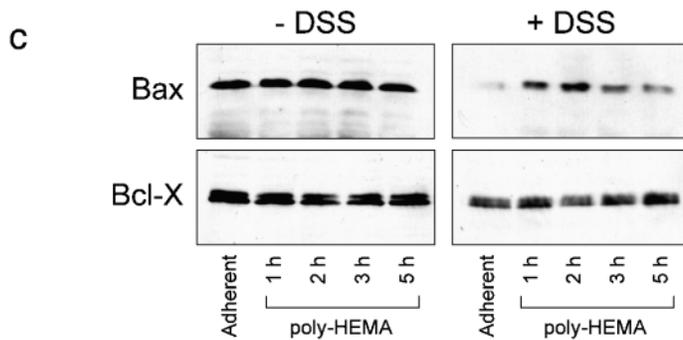
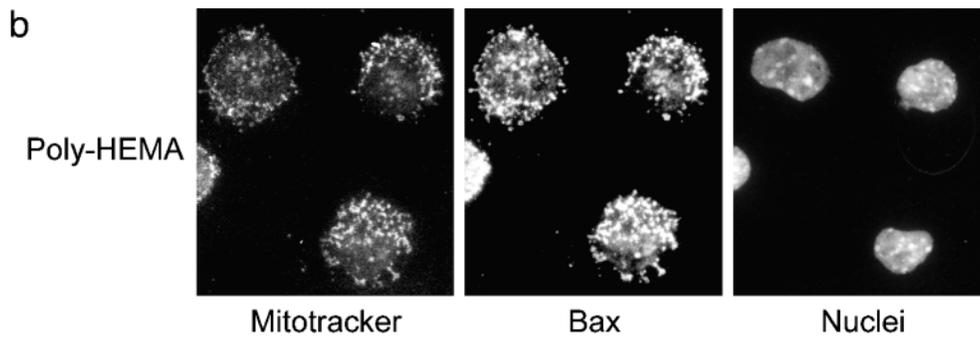
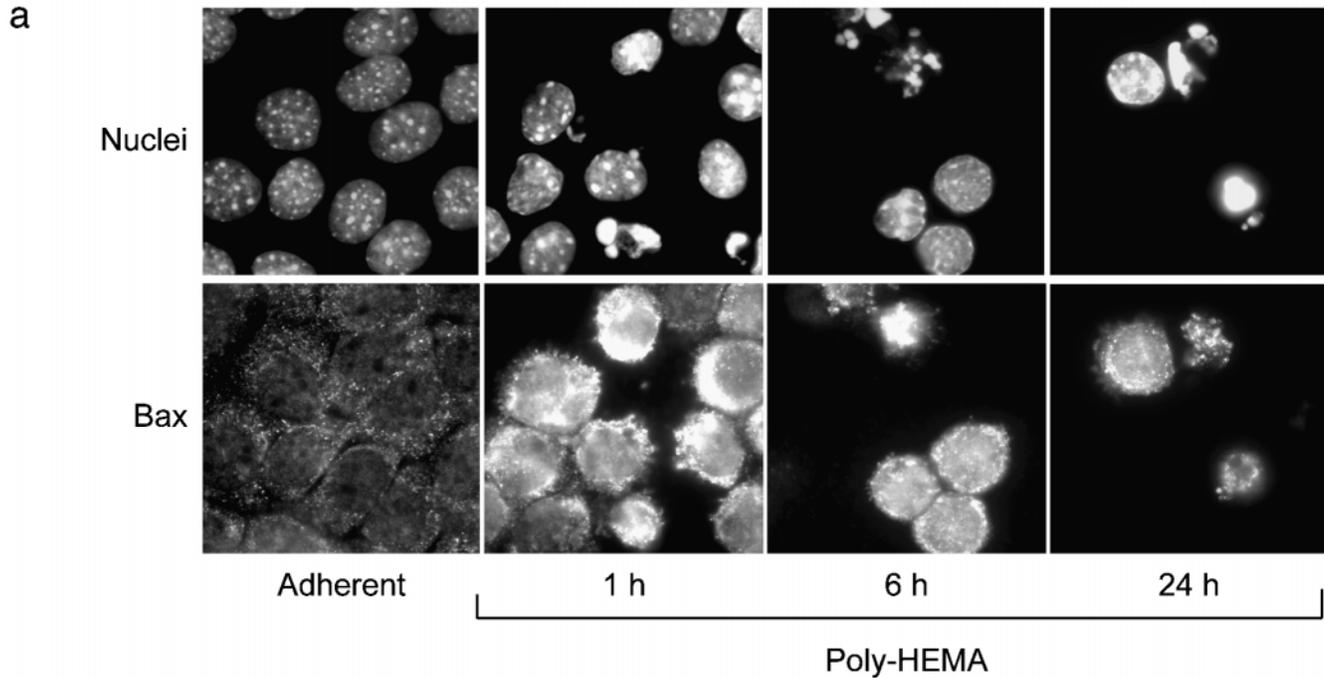
### Bax Redistribution Occurs to Mitochondria

To determine whether Bax translocated to an identifiable subcellular compartment, cells were stained with a polyclonal antibody raised against a peptide corresponding to amino acids 44–59 of Bax (62M) (Metcalf et al., 1999).



were analyzed by immunoblotting with 62M anti-Bax and monoclonal anti-Bcl-x. (d) FSK-7 cells were treated with 25  $\mu$ g/ml cycloheximide for 1 h before detachment from ECM for 3 h. Cells were fractionated and Bax localization analyzed by immunoblotting with 62M.

**Figure 2.** Subcellular distribution of Bax in mammary epithelial cells is altered after detachment from ECM. (a) Adherent FSK-7 cells were either maintained on tissue culture dishes or were detached from ECM and incubated on poly-HEMA for 1 h. Cells were fractionated into insoluble (Insol) and cytosolic (Cyt) fractions as described in Materials and Methods. Fractions were analyzed by immunoblotting for Bax, Bcl-x, calnexin, and cytochrome oxidase subunit I (COX I). Similar results were obtained for primary mammary epithelial cells. Data are representative of multiple experiments. (b) FSK-7 cells were transfected with 2  $\mu$ g of pCDNA.3, pCDNA.3/HA-Bax, or pCDNA.3/HA-Bax $\Delta$ CT. 18 h after transfection cells were stained with Hoechst 33258, anti-HA mAb and 62M anti-Bax. Transfected cells were scored for percent apoptotic cells based on nuclear morphology. (c) FSK-7 cells were detached from ECM and maintained on poly-HEMA for 5 and 12 h before homogenization and separation into insoluble and cytosolic fractions. Fractions



**Figure 3.** Detachment-induced Bax relocalization occurs to mitochondria and coincides with a conformational change that exposes its BH3 domain. (a) Adherent FSK-7 cells and detached cells maintained on poly-HEMA for the indicated times before cytopinning onto polysine slides, were immunostained with anti-Bax 62M and Hoechst 33258. (b) FSK-7 cells on poly-HEMA for 1 h were incubated with Mitotracker before cytopinning and immunostaining for Bax. (c) Adherent cells or cells on poly-HEMA for the indicated times were treated for 30 min with either the cell permeable cross-linker DSS or carrier alone. Total cell lysates were then analyzed by immunoblotting for Bax or Bcl-x. No change in Bcl-x immunoreactivity was observed after DSS treatment. Results are representative of three experiments.

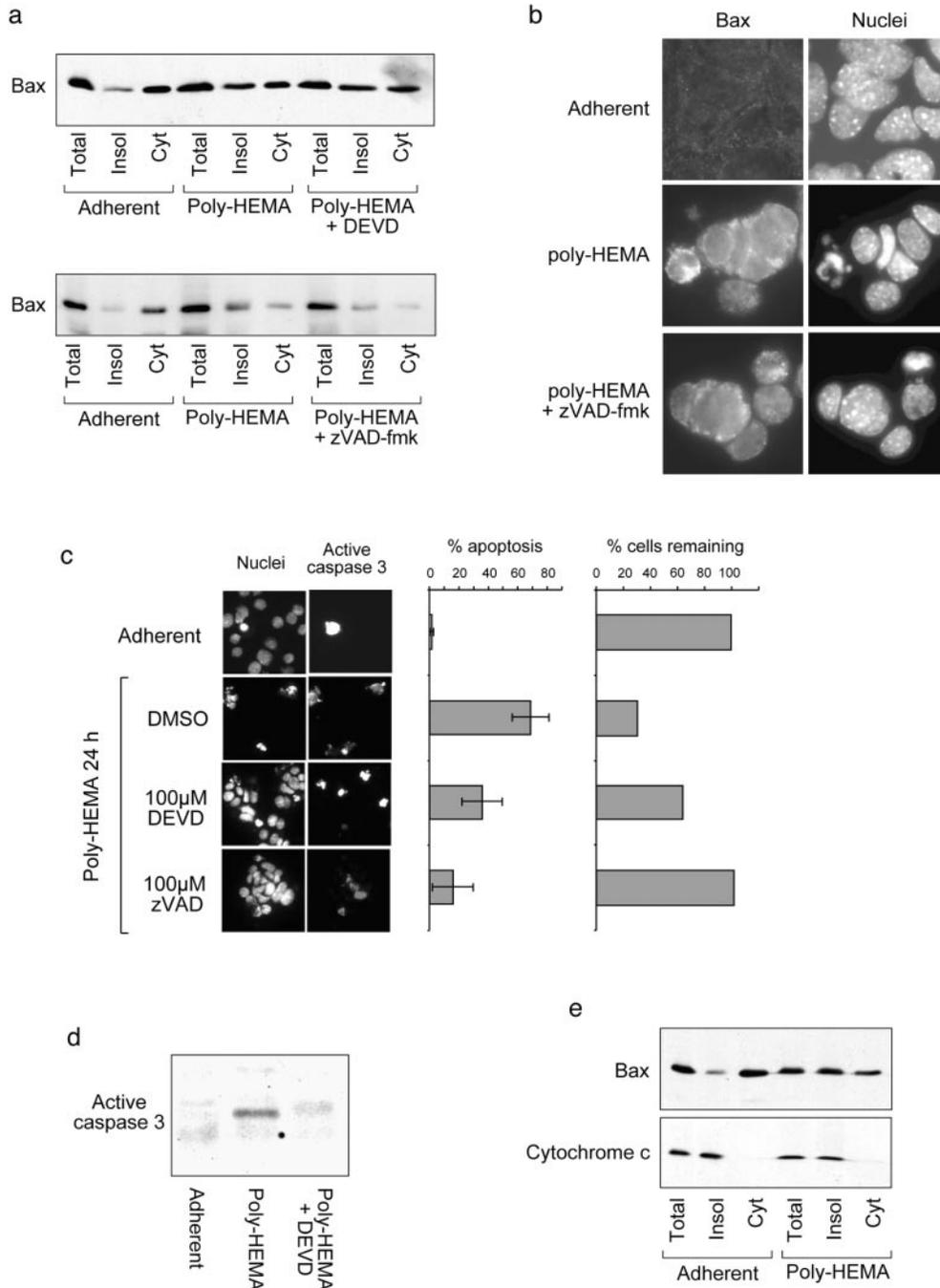
This region of Bax is at its amino terminus and close to the BH3 domain critical for its pro-apoptotic function (Hunter and Parslow, 1996). FSK-7 cells maintained in suspension for 1, 6, and 24 h were cytopun onto polysine-coated slides and Bax staining was compared with that in an adherent monolayer of cells (Fig. 3 a). Detachment from ECM resulted in a marked increase in Bax staining, which

showed a punctate distribution within the cytoplasm. This occurred within 1 h of detachment, on a time course identical with the observed translocation of Bax to the insoluble fraction of the cells, and preceding the onset of apoptosis, seen through the condensation and fragmentation of nuclei at 6 and 24 h (compare Fig. 3 a with 1 b). To determine if Bax localized to mitochondria, detached cells were

incubated with the mitochondrial stain Mitotracker green-fm before fixation and immunostaining with the 62M antibody. This is incorporated into the mitochondria of live cells, but is retained after aldehyde fixation. Bax immunofluorescence in detached cells was found to coincide with Mitotracker staining (Fig. 3 b). The relocation of Bax to an insoluble fraction after detachment from ECM therefore coincided with the exposure of its BH3 domain and its appearance within mitochondria.

To verify that the conformation of the Bax BH3 epitope altered during detachment-induced apoptosis, living FSK-7 cells were treated with the membrane permeable cross-linker DSS. Cells were treated with DSS before lysis, and

compared with untreated cells. In the absence of cross-linking, there was no change in the total amount of Bax present over the time course of the experiment. However, we observed very little Bax immunoreactivity with the 62M antibody in whole cell lysates after cross-linking of proteins within monolayers of cells adherent to ECM (Fig. 3 c). After detachment, however, Bax immunoreactivity increased with a time course similar to that observed both for redistribution of Bax to the insoluble fraction and for the increase in Bax immunofluorescence. A similar result was seen with a second anti-Bax antibody (data not shown). As with Bax translocation, the increase in Bax immunoreactivity after cross-linking was not blocked by treating cells on



**Figure 4.** Detachment-induced Bax redistribution precedes activation of caspases and cytochrome c release. (a) 100  $\mu$ M of the caspase inhibitors, DEVD or zVAD-fmk, were added 1 h before detachment of FSK-7 cells from ECM, and throughout the incubation on poly-HEMA for 3 h. Cells were homogenized in hypotonic buffer and insoluble and cytosolic fractions were analyzed with the 62M anti-Bax antibody. (b) FSK-7 cells with or without caspase inhibitors were maintained on poly-HEMA for 1 h before immunostaining for Bax. (c) FSK-7 cells were maintained on poly-HEMA for 24 h in the presence or absence of either zVAD or DEVD. Cells were cytospun onto glass slides before immunostaining with anti-activated caspase 3 and Hoechst 33258. Both the percentage of apoptotic cells and the number of cells remaining were determined. Caspase inhibitors reduced both the loss of cell number and the percentage apoptosis in the remaining cells. (d) FSK-7 cells were left attached to ECM or detached and maintained in the presence or absence of DEVD. Whole cell lysates were analyzed by immunoblotting with the antibody to activated caspase 3, CMI. (e) FSK-7 cells, either adherent or detached and maintained on poly-HEMA for 3 h, were fractionated and the distribution of cytochrome c compared with Bax.

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poly-HEMA with inhibitors of caspases (data not shown; see below). Together, the results of our immunostaining and cross-linking studies indicate that Bax is normally present in the cytosol but that its BH3 domain is concealed. Loss of mammary epithelial cell interactions with the ECM result in a rapid translocation of Bax to mitochondria, coinciding with a conformational change to expose its BH3 domain.

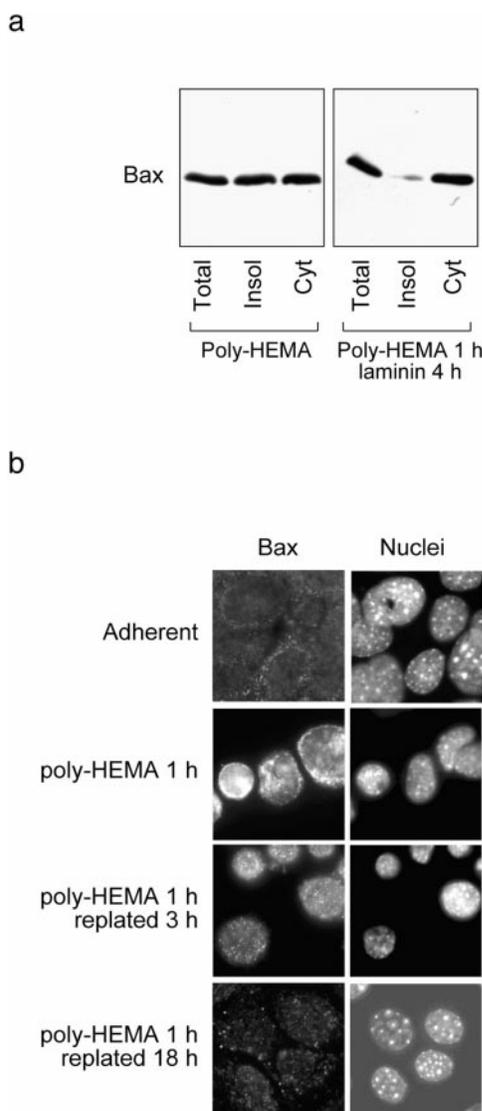
### **Bax Redistribution Occurs before Commitment to Apoptosis and Is Reversible**

The rapidity of Bax redistribution after detachment suggested that it was an event upstream of the execution phase of apoptosis. Treating cells on poly-HEMA with the broad spectrum caspase inhibitor zVAD-fmk, or the caspase 3-like inhibitor DEVD, did not prevent the redistribution of Bax to the insoluble compartment of FSK-7 cells (Fig. 4 a) or the appearance of the BH3 proximal epitope (Fig. 4 b). Caspase inhibitors did, however, reduce detachment-induced apoptosis and inhibit the activation of caspase 3 (Fig. 4, c and d). We also examined the distribution of cytochrome c. Bax can induce cytochrome c release from isolated mitochondria (Jurgensmeier et al., 1998; Narita et al., 1998), and overexpression of Bax can induce cytochrome c release in fibroblasts and melanoma cells (Rosse et al., 1998). However, in hematopoietic cells, Bax association with mitochondria does not result in cytochrome c release, but does result in mitochondrial dysfunction and apoptosis (Wolter et al., 1997; Gross et al., 1998). At 3 h after detachment from ECM, cytochrome c was still associated with the insoluble fraction, even though Bax had redistributed from the cytosol (Fig. 4 e). This also provided further evidence that mitochondrial integrity had not been affected during homogenization. The results indicate that the adhesion-dependent relocation of Bax occurs before caspase activation.

Bax translocation occurred upstream of the execution phase of apoptosis. To determine if it was reversible, FSK-7 cells were detached and maintained on poly-HEMA for 1 h, after which a portion were then replated onto laminin before they had committed to apoptosis. After 1 h in suspension, Bax relocated to the insoluble fraction (Fig. 5 a) and its BH3 domain became exposed (Fig. 5 b). After replating for 4 h, 94.3% ( $\pm 3.9$  SEM,  $n = 3$ ) of the cells reattached and spread, and in those cells Bax redistributed back to the soluble fraction. The extent of BH3 domain exposure determined by Bax immunostaining, diminished after replating for 3 h and was absent after 18 h. To confirm that the reattached cells had not committed to apoptosis, bromodeoxyuridine (BrdU) was included in the growth medium for 24 h, and the cells were fixed and stained with a monoclonal antibody to BrdU. There was no difference between the percentage of replated cells that incorporated BrdU with those constantly maintained on ECM (data not shown). Together, these results indicate that detachment-induced translocation of Bax to the insoluble fraction occurs upstream of commitment to apoptosis and is reversible. Since the adhesion of mammary epithelial cells to laminin is mediated by  $\beta 1$  integrins (Klinowska et al., 1999), our results also demonstrate that Bax translocation and BH3 domain epitope exposure are integrin dependent.

### **Signaling Pathways Associated with Cell Adhesion Regulate Bax Translocation**

The tyrosine phosphorylation of a number of integrin-associated signaling proteins has been shown to be central to adhesion-mediated signal transduction (Howe et al., 1998). We therefore determined whether either the subcellular distribution of Bax or mammary cell apoptosis was regulated by tyrosine kinases. The protein tyrosine kinase inhibitor, herbimycin A prevents adhesion-induced tyrosine phosphorylation of focal adhesion proteins, including pp125FAK and its targets (Burrige et al., 1992). Herbimycin A treatment

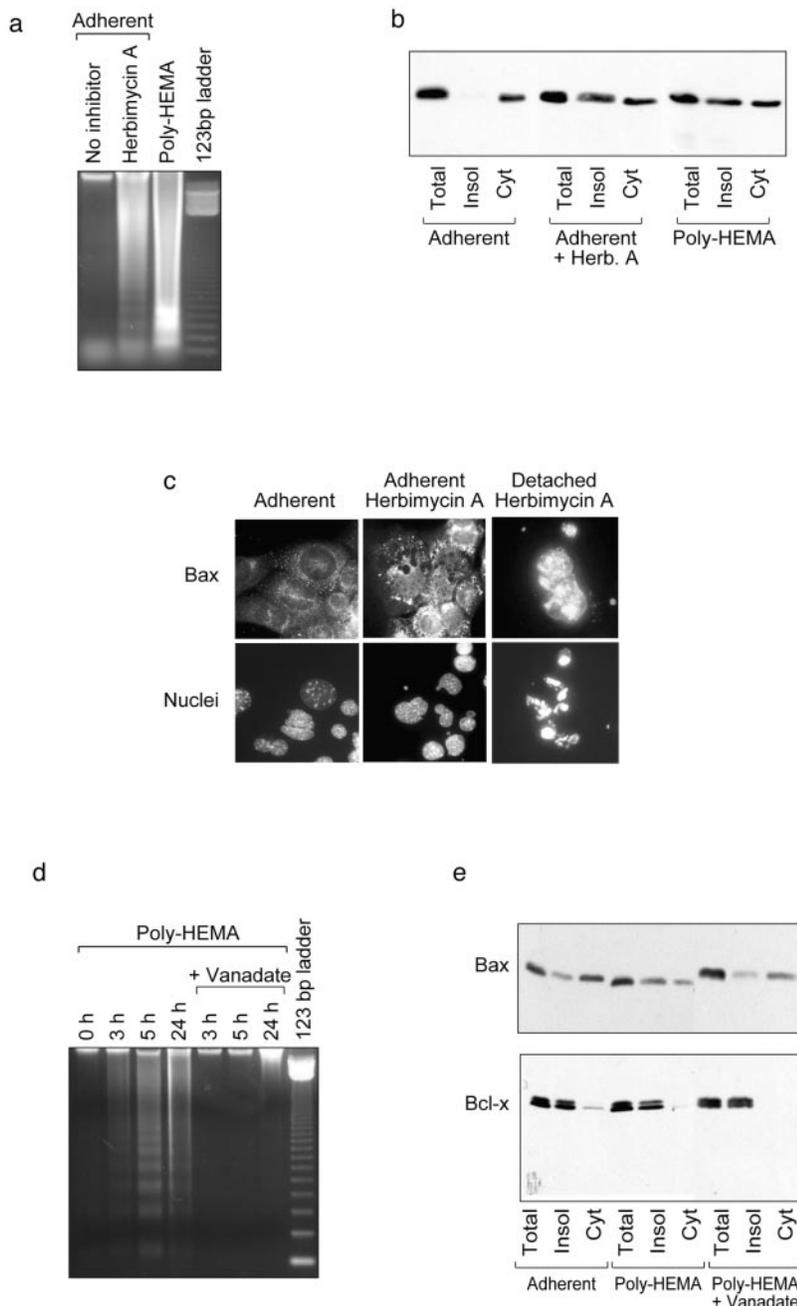


**Figure 5.** Bax relocalization and exposure of its BH3 domain is reversible. (a) Adherent FSK-7 cells were detached from ECM and maintained on poly-HEMA for 1 h. Half were then homogenized and fractionated. The other half were replated onto laminin for 4 h before homogenization. Insoluble and cytosol fractions were analyzed by immunoblotting for Bax. (b) FSK-7 cells were immunostained for Bax. Cells were adherent, detached from ECM for 1 h, or detached for 1 h and then replated onto laminin for 3 or 18 h. Note that Bax staining is diminished 3 h after plating on ECM and is virtually absent after 24 h.

of adherent FSK-7 cells induced apoptosis, seen by the appearance of nucleosomal ladders (Fig. 6 a). It also resulted in translocation of Bax from the soluble to the insoluble fraction (Fig. 6 b), but had no effect on the total amount of expressed Bax or the distribution of Bcl-x. In addition, treatment with herbimycin A for 5 h resulted in an increase in punctate staining of the Bax BH3 epitope, similar to that observed in cells maintained on poly-HEMA (Fig. 6 c). All the attached cells showing increased Bax staining had normal nuclei, whereas all those cells that had detached after 5 h herbimycin A treatment were apoptotic, with condensed and fragmented nuclei, and all showed intense punctate Bax staining. Thus, inhibition of tyrosine kinases in adherent cells induced the exposure of the Bax

BH3 domain epitope and its translocation from the cytosol to the insoluble fraction, and this preceded the onset of apoptosis.

To confirm that protein tyrosine kinases were involved with the adhesion-dependent regulation of Bax translocation and mammary epithelial cell apoptosis, detached cells maintained on poly-HEMA were treated with the protein tyrosine phosphatase inhibitor pervanadate. Pervanadate completely inhibited detachment-induced apoptosis, even after 24 h in suspension (Fig. 6 d). Treatment with pervanadate also prevented Bax translocation to the insoluble fraction after detachment (Fig. 6 e). Similar results were also seen with orthovanadate alone, and in control experiments hydrogen peroxide alone did not suppress apoptosis



**Figure 6.** Adhesion-dependent subcellular localization of Bax and apoptosis are regulated by protein tyrosine kinases. (a) Adherent FSK-7 cells were treated with 1  $\mu$ M herbimycin A in DMSO or with DMSO alone, or were maintained on poly-HEMA. Cells were treated for 24 h before harvesting and extracting low molecular mass DNA for analysis of nucleosomal ladders. (b) Adherent FSK-7 cells were treated with 1  $\mu$ M herbimycin A or carrier alone, or maintained on poly-HEMA, for 3 h. Cells were then lysed in hypotonic buffer and fractionated by Dounce homogenization. Bax localization was analyzed by immunoblotting. Treatment with herbimycin A resulted in an increase in the amount of Bax associated with the insoluble fraction. (c) Adherent FSK-7 cells were treated with 1  $\mu$ M herbimycin A for 5 h before fixation in 3.7% formaldehyde and staining with anti-Bax 62M and Hoechst 33258 to visualize nuclei. Cells treated with carrier alone only showed weak staining with 62M. Herbimycin A-treated cells showed an increase in the punctate Bax staining as seen in detached FSK-7 cells (compare with Figs. 3 and 4). Treatment of cells with herbimycin A resulted in a number of cells detaching from the monolayer. These cells were cytospun onto polysine slides and stained for Bax. All displayed condensed and fragmented nuclei, typical of apoptosis, and stained strongly for Bax. (d) FSK-7 cells were detached and maintained on poly-HEMA with or without pervanadate. Cells were harvested at 3, 5, and 24 h. Low molecular mass DNA was isolated and separated on 1.2% agarose to assay nuclear laddering. Pervanadate completely inhibited the appearance of DNA ladders, even after 24 h culture on poly-HEMA. (e) Cells cultured either as an adherent monolayer, or as detached cells on poly-HEMA with or without pervanadate, were lysed in hypotonic buffer and fractionated into insoluble and cytosolic fractions. Fractions were separated by SDS-PAGE and analyzed by immunoblotting with anti-Bax 62M and anti-Bcl-x antibodies. Pervanadate-treated cells still show predominantly soluble Bax. Note the apparent reduction in mobility of Bax after pervanadate treatment, which is not seen with Bcl-x.

(data not shown). These results suggest that Redox effects were not responsible for inhibition of apoptosis. Interestingly, Bax from pervanadate-treated cells also demonstrated a slight but reproducible decrease in mobility as seen by SDS-PAGE and immunoblotting. This was not due to direct tyrosine phosphorylation of Bax, as Bax was not detectable using anti-phosphotyrosine antibodies, neither was Bax phosphorylation detected in immunoprecipitates from  $^{32}\text{P}$ -orthophosphate labeled cells. The cause for this mobility shift is, therefore, not yet clear. Pervanadate treatment had no effect on the distribution of Bcl-x or its mobility on SDS-PAGE. Together, these results indicate that protein tyrosine kinases are central in mediating adhesion-regulated subcellular localization of Bax and apoptosis.

### ***Bax Localization and Conformation Is Regulated by pp125FAK***

Pp125FAK has previously been reported to regulate adhesion-dependent cell survival in serum starved fibroblasts (Hungerford et al., 1996; Ilic et al., 1998). To determine if inhibition of pp125FAK signaling in mammary epithelial cells resulted in apoptosis and altered exposure of the Bax BH3 epitope, its activity was inhibited by using dominant negative FAK constructs. A dominant negative pp125FAK, consisting of its carboxy-terminal region fused to glutathione-S-transferase (DN-FAK), contains the focal adhesion-targeting sequence of pp125FAK but lacks the kinase domain and the critical tyrosine phosphorylation site at Tyr-397 required for interaction with src and PI 3-kinase. This construct has previously been shown to displace endogenous pp125FAK from the focal adhesions of fibroblasts and endothelial cells (Gilmore and Romer, 1996).

To ascertain whether or not pp125FAK was required for survival in mammary epithelia, FSK-7 cells were transfected with either DN-FAK or pCDNA.3 alone, and the number of apoptotic cells determined 24 h later. DN-FAK induced almost five times the amount of apoptosis seen with vector alone, and this was inhibited with zVAD-fmk (Fig. 7 a). To examine whether DN-FAK regulated Bax conformation, transfected cells were immunostained using the BH3 proximal antibody (62M). DN-FAK-expressing cells that showed apoptotic morphology stained strongly with 62M, whereas there was little immunoreactivity in nonexpressing cells with healthy nuclei (Fig. 7 b). 24 h after transfection, DN-FAK-expressing cells that had undergone apoptosis had mostly detached, and these cells were cytopun before immunostaining. Virtually all detached apoptotic cells stained positive both for DN-FAK and the 62M anti-Bax antibody. DN-FAK-expressing cells also stained strongly with the activated caspase 3 antibody (data not shown).

Using transfections, we were unable to examine the time course of Bax epitope exposure after inhibition of pp125FAK signaling. Therefore, to address early events after inhibition of pp125FAK signaling, DN-FAK protein was microinjected into FSK-7 cells. Cells were then assayed at 1 and 5 h postinjection (Fig. 7 c). FSK-7 cells injected with DN-FAK showed a marked inhibition of phosphotyrosine staining in focal adhesions 1 h after injection compared with noninjected cells and to cells injected with

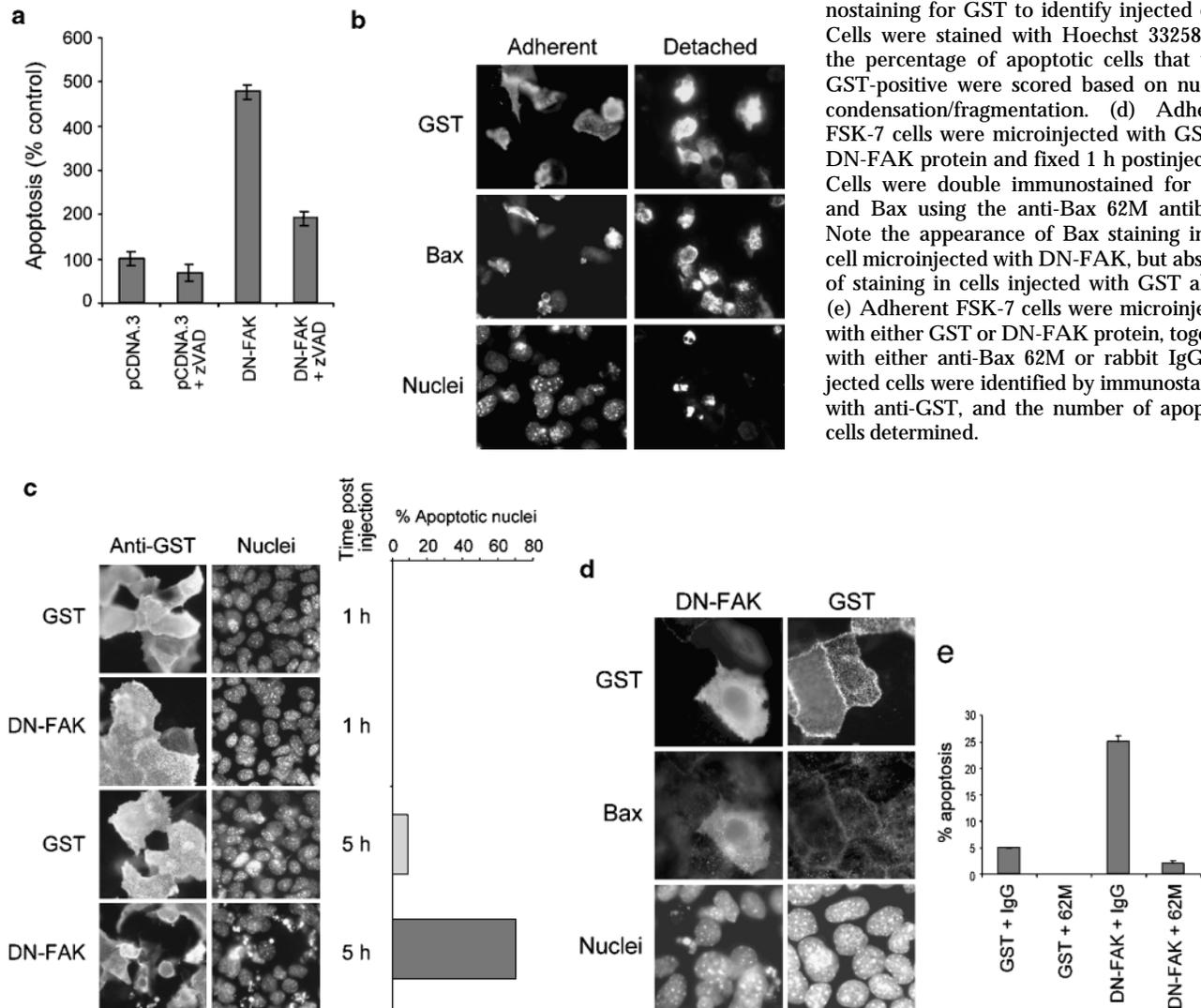
the GST control (data not shown). At 5 h postinjection, there was an increase in the proportion of DN-FAK-injected cells with apoptotic morphology, whereas very few GST-injected cells showed abnormal morphology (Fig. 7 c). Not all the DN-FAK-injected cells became apoptotic after 5 h, possibly due to each cell receiving different doses of the fusion protein, as was found previously in endothelial cells and fibroblasts where the DN-FAK inhibitory effect was highly dose dependent (Gilmore and Romer, 1996). We also examined exposure of the Bax BH3 epitope after DN-FAK injection. Immunostaining using the 62M antibody increased in a proportion of DN-FAK-injected cells within 1 h of injection (Fig. 7 d). This staining showed a similar pattern of staining to that seen in FSK-7 cells on poly-HEMA, with a punctate distribution. These results demonstrate that injection of DN-FAK protein in adherent mammary cells leads to rapid exposure of its BH3 epitope within 1 h, followed by the induction of apoptosis in a high proportion of cells within 5 h. Thus in adherent cells, pp125FAK is required to maintain survival and inhibiting its function results in rapid Bax BH3 domain exposure, followed by apoptosis.

To determine whether Bax was required for DN-FAK-induced apoptosis, the BH3 proximal antibody 62M was coinjected into FSK-7 cells with the fusion proteins. Cells were then examined 5 h post injection. Whereas DN-FAK-induced cell death when coinjected with nonspecific rabbit IgG, there was no loss of cells when injected together with 62M (Fig. 7 e). Thus, antibody binding near the critical BH3 domain required for apoptosis prevented Bax apoptotic function. Injection with either antibody (IgG or 62M) together with GST had no significant effect on apoptosis. These results provide evidence that the exposure of the Bax BH3 epitope is required for apoptosis induced by inhibiting pp125FAK signaling.

### ***Pp125FAK Mediates Apoptosis through PI 3-Kinase and pp60src***

Pp125FAK can activate a number of downstream signaling pathways (Hanks and Polte, 1997). PI 3-kinase and pp60src both bind to pp125FAK (Chen and Guan, 1994; Cobb et al., 1994) and have been implicated in survival signaling (Khwaja et al., 1997; McGill et al., 1997). To determine if pp125FAK mediated mammary cell survival through either PI 3-kinase or pp60src, DN-FAK was cotransfected with either the constitutively active p110 subunit of PI 3-kinase (p110CAAX) or with activated pp60src. Both active PI 3-kinase and pp60src blocked DN-FAK-induced apoptosis (Fig. 8 a). Previously, pp125FAK has been shown to promote survival of primary fibroblasts through a PKC- and p53-dependent pathway and not through a PI 3-kinase route (Ilic et al., 1998). To confirm that PI 3-kinase was required for MEC survival, FSK7 cells were transfected with a dominant negative p85 subunit (p85 $\Delta$ SH2). This induced apoptosis as efficiently as DN-FAK (Fig. 8 a). We examined the exposure of the Bax BH3 domain in p85 $\Delta$ SH2-transfected cells. Cells expressing p85 $\Delta$ SH2 stained strongly with the 62M antibody and showed apoptotic nuclei, whereas healthy untransfected cells were negative for Bax BH3 epitope exposure (Fig. 8 b). The p85 $\Delta$ SH2-expressing cells that had detached after apoptosis were also examined after cytopinning, and

**Figure 7.** Mammary epithelial cell apoptosis and Bax BH3 epitope exposure is regulated by pp125FAK. (a) FSK-7 cells were transfected with either DN-FAK or vector alone, in the presence of either 100  $\mu$ M zVAD or carrier alone. 24 h posttransfection, the number of apoptotic cells were determined by Hoechst staining. (b) DN-FAK-transfected FSK-7 cells were immunostained 24 h posttransfection for GST and Bax using anti-Bax 62M. Both adherent cells and cells that had detached after apoptosis were stained. DN-FAK-expressing cells showing Bax staining were apoptotic, whereas all nonexpressing cells showed healthy nuclei and were negative for Bax immunoreactivity (examples of healthy nontransfected cells are indicated by arrows). All detached cells were Bax positive and had apoptotic nuclei. (c) Adherent FSK-7 cells were microinjected with either 3 mg/ml GST or GST-tagged DN-FAK protein. Cells were fixed 1 and 5 h postinjection, followed by immunostaining for GST to identify injected cells. Cells were stained with Hoechst 33258 and the percentage of apoptotic cells that were GST-positive were scored based on nuclear condensation/fragmentation. (d) Adherent FSK-7 cells were microinjected with GST or DN-FAK protein and fixed 1 h postinjection. Cells were double immunostained for GST and Bax using the anti-Bax 62M antibody. Note the appearance of Bax staining in the cell microinjected with DN-FAK, but absence of staining in cells injected with GST alone. (e) Adherent FSK-7 cells were microinjected with either GST or DN-FAK protein, together with either anti-Bax 62M or rabbit IgG. Injected cells were identified by immunostaining with anti-GST, and the number of apoptotic cells determined.



these all showed Bax epitope exposure (Fig. 8 b). Similarly, treatment of FSK-7 cells with the PI 3-kinase inhibitor wortmannin resulted in apoptosis and translocation of Bax to the insoluble fraction (data not shown). Bax epitope exposure was inhibited in cells that had been cotransfected with DN-FAK and either p110CAAX or src, consistent with the hypothesis that signaling through PI 3-kinase and src linked pp125FAK with suppression of Bax activation (Fig. 8 c). Together with the fibroblast study (Ilic et al., 1998), these results indicate that pp125FAK might suppress apoptosis using cell type-specific signaling pathways. We suggest that in mammary epithelial cells, the activation of pp125FAK by integrin-mediated adhesion to ECM may act through downstream kinases such as PI 3-kinase to regulate the conformation and subcellular distribution of Bax, thereby controlling apoptotic fate.

## Discussion

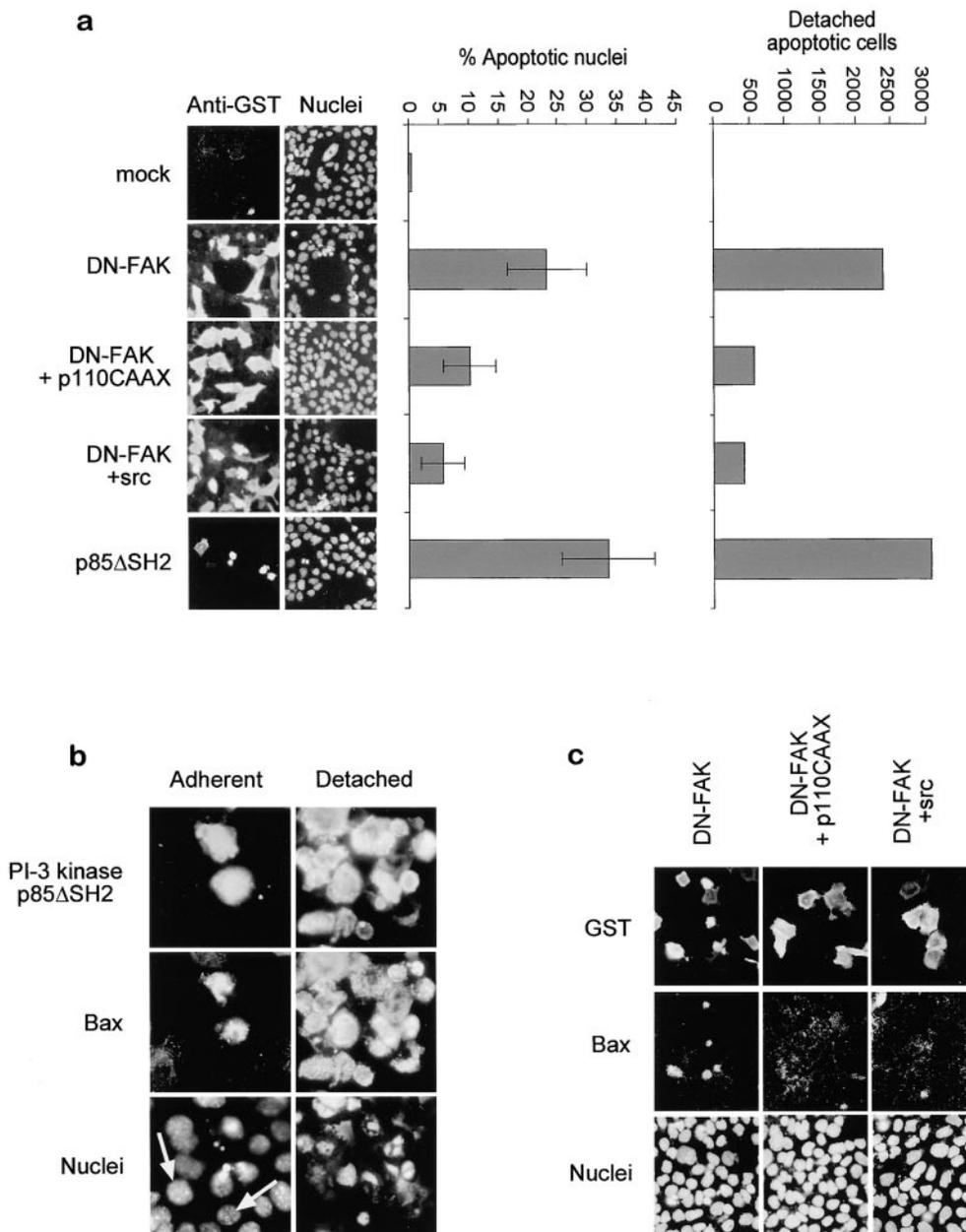
ECM is an important determinant for epithelial cell survival (Frisch and Francis, 1994; Boudreau et al., 1995; Khwaja et al., 1997; Farrelly et al., 1999), but until now the components that link adhesion to the apoptotic machinery have not been identified. In this paper, we demonstrate that detachment of epithelial cells from ECM results in reversible changes in the conformation and localization of Bax before the onset of apoptosis. We have identified early decision making events in this process, as ECM interactions suppress apoptosis through signaling pathways activated by the integrin-associated kinase pp125FAK. Our results suggest that pp125FAK, through PI 3-kinase and pp60src, maintains Bax in a conformation that prevents its relocation to mitochondria. Bax translocation is upstream

of caspase activation, indicating that it is an early event after inhibition of adhesion-dependent survival signals.

### Survival Signaling in Mammary Epithelial Cells Is Mediated through pp125FAK

Most cells require adhesion to ECM for survival. This has now been demonstrated for primary cultures of endothelial, epithelial, fibroblastic, mesangial, muscle, and neuronal cells. Furthermore, survival signaling in these cell systems is mediated through integrins (Meredith et al., 1993; Frisch and Francis, 1994; Vachon et al., 1996; Ilic et

al., 1998; Farrelly et al., 1999; Frost et al., 1999; Mooney et al., 1999). Here we show that mammary cells undergo extremely rapid apoptosis after complete detachment from the ECM. Our previous work has shown that long-term survival of mammary epithelial cells in culture requires integrin-mediated interactions with a laminin-rich basement membrane. Monolayers of cells on collagen I or on tissue culture plastic undergo apoptosis over a period of several days (Pullan et al., 1996; Farrelly et al., 1999). Thus, even though collagen I does not provide as efficient survival signals as laminin, it does afford some protection to apoptosis compared with complete detachment from ECM. In both



**Figure 8.** The p110 subunit of PI 3-kinase and pp60src act downstream of pp125FAK in adhesion-mediated apoptosis suppression. (a) FSK-7 cells were transfected with 2  $\mu$ g pCDNA.3 (mock), 2  $\mu$ g pCDNA.3/DN-FAK (DN-FAK), 2  $\mu$ g pCDNA.3/DN-FAK + 1  $\mu$ g pCDNA.3/p110CAAX (DN-FAK + p110CAAX), 2  $\mu$ g pCDNA.3/DN-FAK + 1  $\mu$ g pCDNA.3/src (DN-FAK + src), and 2  $\mu$ g pCDNA.3/p85 $\Delta$ SH2 (p85 $\Delta$ SH2). 24 h after transfection the adherent cells were stained with Hoechst 33258 and either anti-GST or anti-p85 (for the p85 $\Delta$ SH2-transfected cells), and the percentage of adherent transfected cells were scored for apoptosis. Detached cells were cytopspun and the number of cells expressing DN-FAK or p85 $\Delta$ SH2 were counted. (b) FSK-7 cells transfected with pCDNA.3/p85 $\Delta$ SH2 were immunostained 24 h posttransfection with anti-p85 and anti-Bax 62M, as well as Hoechst 33258 to visualize nuclei. P85 $\Delta$ SH2-expressing cells were positive for Bax immunoreactivity, as well as showing apoptotic nuclei, in contrast to nonexpressing cells which were Bax negative and had healthy nuclei (examples of healthy cells are indicated by arrows). Detached apoptotic cells were cytopspun before staining, and all showed p85 and Bax BH3 epitope immunoreactivity. (c) FSK-7 cells were cotransfected with DN-FAK and p110CAAX or src as in a, 24 h later, cells were fixed and immunostained for GST and Bax, as well as Hoechst 33258.

cases, apoptosis occurs even in the presence of serum growth factors, including insulin and EGF, demonstrating the primal nature of the ECM-derived survival signal.

Pp125FAK represents an excellent candidate signaling protein for transducing adhesion-mediated survival signals. It has been shown to be a key apoptosis regulator in several cell types including fibroblasts, human cancer cell lines, and MDCK epithelial cells (Frisch et al., 1996; Hungerford et al., 1996; Ilic et al., 1998; Xu et al., 1998; Sonoda et al., 1999). Our work now implicates an essential role for pp125FAK in mammary cell survival since both microinjected DN-FAK protein and transfected DN-FAK expression vectors induce apoptosis rapidly. Although we cannot totally discount the possibility that DN-FAK disrupts cell adhesion, and thus induces apoptosis, we think this is unlikely. First, DN-FAK did not disrupt focal adhesions when microinjected into fibroblasts and primary endothelial cells, although it did block downstream signaling (Gilmore and Romer, 1996). Second, the number of detached cells resulting from DN-FAK transfection was reduced by treatment with zVAD-fmk, indicating that detachment was due to apoptosis rather than disruption of adhesion by DN-FAK. Third, activated mutants of PI 3-kinase and src also reduced the number of cells detaching after DN-FAK transfection. Together these observations suggest that detachment follows apoptosis in DN-FAK-expressing cells and not vice versa.

A variety of downstream effectors may propagate survival signals from pp125FAK, as it lies in a nodal position for effecting integrin-dependent cellular responses and can potentially act through PI 3-kinase, pp60src, as well as adapter molecules such as Grb2, paxillin and p130CAS (Chen and Guan, 1994; Schlaepfer et al., 1994; Schaller and Parsons, 1995; Cary et al., 1996; Chen et al., 1996; Cary et al., 1998; Thomas et al., 1998). One candidate signaling kinase is PI 3-kinase, which regulates apoptosis in kidney epithelial cells as well as in neuronal and hematopoietic cells (Minshall et al., 1996; Dudek et al., 1997; Khwaja et al., 1997; Philpott et al., 1997; Vaillant et al., 1999). A potential role for PI 3-kinase in adhesion-mediated survival signaling comes from experiments where the expression of constitutively activated PI 3-kinase as well as one of its substrates, protein kinase B, protects MDCK cells from apoptosis in suspension (Khwaja et al., 1997), and where adhesion to fibronectin directly results in the accumulation of PI 3-kinase lipid products as well as p85-dependent protein kinase B activity (King et al., 1997). In the mammary system we have previously demonstrated that apoptosis is induced by pharmacological inhibitors of PI 3-kinase (Farrelly et al., 1999), and now show that cell death results from expression of the dominant negative p85 subunit of PI 3-kinase, whereas apoptosis induced by DN-FAK is suppressed by the constitutively active p110 subunit of PI 3-kinase. Since adhesion to ECM leads to the association of PI 3-kinase with pp125FAK and its activation in several cell types (Chen and Guan, 1994; Chen et al., 1996; King et al., 1997), including mammary epithelial cells (our unpublished data), it would seem to be a likely downstream effector of pp125FAK-mediated survival signaling.

However, the plethora of binding sites for signaling molecules on FAK suggests that it may regulate survival through different mechanisms in different cell types. This

is borne out at both the cellular and molecular levels, as detachment of fibroblasts from ECM, for example, does not lead to rapid apoptosis induction as is the case for epithelia (Frisch and Francis, 1994). In serum-starved rat synovial fibroblasts, DN-FAK induced apoptosis, but this was not reversed by activating the PI 3-kinase pathway (Ilic et al., 1998). In this cell type, pp125FAK acted through a p53-dependent pathway involving the  $\lambda/\iota$  isoform of protein kinase C and phospholipase A2. Further experiments showing that PI 3-kinase inhibition did not induce apoptosis suggests that fibroblasts may be independent of PI 3-kinase for survival. Thus, whereas PI 3-kinase is important in epithelia, it may not be a primary determinant of adhesion-mediated survival in other cell types such as synovial fibroblasts.

Other pp125FAK-mediated pathways than PI 3-kinase may also be involved with epithelial cell survival. Constitutively active pp125FAK can suppress apoptosis in MDCK cells detached from ECM, but only if its kinase domain and its major autophosphorylation site, Y-397, are intact (Frisch et al., 1996). Y-397 binds src family kinases in addition to PI 3-kinase. This association has been proposed to regulate the phosphorylation of cellular substrates through the recruitment of src to focal adhesions (Schaller et al., 1999). In mammary cells we found that activated pp60src also suppressed the apoptotic effects of DN-FAK. Furthermore, transformation by src has been implicated in reducing the sensitivity of fibroblasts to detachment-induced apoptosis (McGill et al., 1997). Whether or not PI 3-kinase and pp60src trigger similar or independent downstream pathways leading to apoptosis suppression is not yet clear. Pp125FAK effects on cell proliferation, through Erk activation, appear to be regulated by Y-397 binding to src and fyn kinase, whereas its regulation of migration also requires an association with p130CAS through one of two carboxy-terminal proline rich domains (Cary et al., 1998; Zhao et al., 1998). Recently, it has been suggested that pp125FAK can activate Jun amino-terminal kinase through a mechanism not requiring pp125FAK kinase activity, but involving an interaction with paxillin through its extreme carboxy-terminal region (Igishi et al., 1999). Thus, the many interactions of pp125FAK with downstream signaling pathways might permit several mechanisms for regulating apoptosis.

### *Linking Integrin Signals to the Apoptosis Machinery*

Adhesion provides an essential anti-apoptotic signal for epithelial cells, yet the mechanism by which it feeds into the apoptotic machinery has not been elucidated. We demonstrate in this paper that whatever the proximal survival pathway regulated by pp125FAK, adhesion regulates apoptosis through a Bax-dependent mechanism.

Previously, integrin-mediated adhesion has been shown to upregulate expression of Bcl-2 in CHO cells, which was proposed to lead to survival according to the rheostat model (Zhang et al., 1995). However, in mammary epithelia Bcl-2 is absent and we have seen no changes in the levels of expression of Bax or Bcl-x either after detachment from matrix or during culture on different ECM substrata (Pullan et al., 1996). Thus, at least in mammary epithelia, altered ratios of these proteins do not seem to regulate apoptosis.

Instead, we find that cell-ECM interactions control Bax translocation between the cytosol and mitochondria. Although Bax is a pro-apoptotic death effector, we argue that adhesion-dependent signaling maintains Bax in the cytosol where it is unable to trigger apoptosis. In response to lack of integrin-mediated survival signals, Bax translocates to a cellular environment where it can initiate apoptosis. Although Bax has been found constitutively associated with internal cell membranes in some cell types (Desagher et al., 1999), GFP-Bax chimeras had a predominantly cytosolic distribution in Cos-7 cells and in other cell types Bax was cytosolic until receipt of a death stimulus, at which point redistribution to mitochondria occurred (Wolter et al., 1997; Goping et al., 1998; Gross et al., 1998).

In common with all but the BH3-only proteins in the Bcl-2 family, Bax has a hydrophobic carboxy-terminal tail of ~20 amino acids. This tail contains a mitochondrial-targeting domain, and directs unrelated peptides to mitochondria (Nguyen et al., 1993; Wolter et al., 1997; Nechushtan et al., 1999). Targeting of Bax to internal membranes appears to be critical for its ability to induce apoptosis, as deletion of the hydrophobic tail abrogates the ability of Bax to induce apoptosis not only in our experiments with mammary epithelia but also in Cos-7 cells and L929 fibroblasts (Wolter et al., 1997). Thus, regulation of Bax targeting to internal cell membranes may be a critical regulatory step in apoptosis.

Concomitant with the translocation of Bax from the cytosol to mitochondria is a conformational change that may be important for its regulation. In hematopoietic cells the amino terminus of Bax is inaccessible to a monoclonal antibody when the protein is in a cytosolic form but becomes exposed during translocation associated with an apoptotic stimulus (Hsu et al., 1997). In our studies an antibody recognizing an epitope just amino-terminal to its BH3 domain becomes exposed after cell detachment from ECM or upon expression of DN-FAK in adherent cells. The Bax BH3 domain is necessary for the induction of apoptosis, as peptides resembling this domain have apoptosis-inducing ability, and substitution of the Bcl-2 BH3 region with that of Bax converts Bcl-2 into a pro-apoptotic protein (Hunter and Parslow, 1996; Cosulich et al., 1997). This would indicate that masking of the BH3 domain is required to maintain survival in healthy cells, but its exposure after an apoptotic stimulus may be an essential part of the commitment process. This suggestion is supported by our own experiments, where coinjection of DN-FAK with the Bax BH3 proximal antibody inhibited the apoptotic effect of the fusion protein. The result directly implicates control of BH3 domain exposure with adhesion-mediated survival signals. The related molecule Bak, shows similar changes in antibody reactivity after treatment of lymphoma cells with DNA damaging agents (Griffiths et al., 1999). However, in this case, although Bak undergoes a conformational change to expose an amino-terminal domain during the early stages of apoptosis commitment, it is constitutively associated with mitochondria. Conformational changes associated with the exposure of the death-inducing BH3 domain may therefore be a common mechanism of activation within the Bcl-2 family in response to a wide variety of cellular insults.

It not yet resolved how Bax conformation and subcellu-

lar localization are controlled, but both its amino and carboxyl termini have been implicated. Deletion of the amino terminus results in dysregulated translocation of in vitro-translated Bax to purified mitochondria, whereas replacing the carboxyl terminus of Bax with the corresponding sequence of Bcl-2, which is constitutively targeted to membranes, also results in constitutive targeting of Bax (Goping et al., 1998). A further point of regulation may be through phosphorylation, although it is unclear whether Bax can be directly phosphorylated. We did not detect phosphorylated Bax in mammary cells radiolabeled with <sup>32</sup>P-orthophosphate, even though other phosphoproteins were readily identified. The BH3-only protein Bad, for example, appears to be suppressed in its apoptosis-inducing ability by IL-3-mediated phosphorylation of S-112 and S-136, which provides binding sites for the chaperone protein 14-3-3. After IL-3 withdrawal, Bad becomes dephosphorylated and translocates to mitochondria (Zha et al., 1996b; Datta et al., 1997). Bax expressed in *E. coli* can be phosphorylated on serine residues by both protein kinase A and MAP kinase (Lewis et al., 1998), although it is not yet known whether this has relevance to Bax regulation in vivo. We found that tyrosine kinase inhibitors induced Bax translocation to mitochondria and BH3 domain epitope exposure in mammary epithelia. Likewise, the protein kinase C inhibitor staurosporine, induced relocalization of Bax in Cos-7 cells (Wolter et al., 1997). It will therefore be important to determine whether kinase-mediated Bax localization occurs through direct or indirect means.

It remains possible that Bax function is regulated by a chaperone. Intriguingly, in vitro-translated Bax was unable to associate with mitochondria unless it had been exposed to a cell lysate derived from apoptotic cells (Goping et al., 1998). Bax does not contain consensus 14-3-3-binding sites, and in our own experiments Bax did not associate with 14-3-3 (unpublished data), which would implicate the involvement of other proteins. One possibility is the BH3-only protein, Bid, although this is unlikely in mammary epithelia as we have been unable to detect it in FSK-7 cells (unpublished data). Bid resides in the cytoplasm of healthy cells and does not induce apoptosis, but after cleavage by caspase 8, which is activated by death receptors such as TNFR and FAS, Bid was found to bind Bax and induce exposure of the Bax amino-terminal region (Green, 1998; Desagher et al., 1999). This suggests that in some cell types, Bid can regulate Bax activity. Recently, caspase 8 has been shown to be activated after MDCK cell detachment from ECM (Frisch, 1999; Rytomaa et al., 1999). However, it is not clear whether Bax activation is dependent upon caspase 8 since zVAD, which inhibits caspase 8, blocked neither Bax translocation to mitochondria in mammary cells in our studies, nor the appearance of Bax immunoreactivity in HeLa cells treated with staurosporine (Desagher et al., 1999). Many questions remain to be answered regarding the precise control of pro-apoptotic Bcl-2 proteins, and it is quite possible that this regulation shows distinct tissue specific variations.

In summary, we have now identified one potential mechanism whereby adhesion can regulate commitment to apoptosis in epithelial cells. Integrin-mediated positional control is required for maintaining homeostasis, and defective integrin signals lead normal cells to commit apoptosis. By alter-

ing the level or activity of integrin-regulated enzymes that control apoptosis, such as pp125FAK, PI 3-kinase and pp60src, malignant cells may subvert positional information and survive in inappropriate environments. Thus, therapeutic intervention at specific or multiple points on this pathway might restore both the Bax dynamics and the mitotic/apoptotic balance in human cancer.

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