Early Events in the Anoikis Program Occur in the Absence of Caspase Activation*

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Adhesion of many cell types to the extracellular matrix is essential to maintain their survival. In the absence of integrin-mediated signals, normal epithelial cells undergo a form of apoptosis termed anoikis. It has been proposed that the activation of initiator caspases is an early event in anoikis, resulting in Bid cleavage and cytochrome c release from mitochondria. We have previously demonstrated that the loss of integrin signaling in mammary epithelial cells results in anoikis and that this is dependent upon translocation of Bax from the cytosol to the mitochondria. In this paper, we ask whether caspases are required for Bax activation and the associated changes within mitochondria. We show that Bax activation occurs extremely rapidly, within 15 min after loss of integrin-mediated adhesion to extracellular matrix. The conformational changes associated with Bax activation are independent of caspases including the initiator caspase-8. We also examined downstream events in the anoikis program and found that cytochrome c release occurs after a delay of at least 1 h, with subsequent activation of the effector caspase-3. This delay is not due to a requirement for new protein synthesis, since cycloheximide has no effect on the kinetics of Bax activation, cytochrome c release, caspase-3 cleavage, or apoptosis. Together, our data indicate that the cellular decision for anoikis in mammary epithelial cells occurs in the absence of caspase activation. Moreover, although the conformational changes in Bax are rapid and synchronous, the subsequent events occur stochastically and with considerable delays.

Apoptosis is an important cellular mechanism for packaging and removing unwanted, damaged, or infected cells (1). Normal cells are kept alive through the action of signal transduction pathways triggered by extracellular ligands but undergo apoptosis if survival factors are withdrawn or if a cell detects inappropriate stress or damage. Apoptosis is mediated by dynamic changes within Bcl-2 family proteins that lead to alterations in mitochondrial homeostasis and activation of the apoptosome. Certain cells may alternatively enter an extrinsic apoptosis program if they are targeted by cells of the immune system, through ligand binding of death domain-containing receptors. This delay is not due to a requirement for new protein synthesis, since cycloheximide has no effect on the kinetics of Bax activation, cytochrome c release, caspase-3 cleavage, or apoptosis. Together, our data indicate that the cellular decision for anoikis in mammary epithelial cells occurs in the absence of caspase activation. Moreover, although the conformational changes in Bax are rapid and synchronous, the subsequent events occur stochastically and with considerable delays.

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The abbreviations used are: ECM, extracellular matrix; MDCK, Madin-Darby canine kidney; z-VAD, benzyloxycarbonyl-Val-Ala-Asp; fnk, fluoromethyl ketone; poly-HEMA, polyhydroxyethylmethacrylate; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid; FACS, fluorescence-activated cell sorting; BH3, Bcl2 homology domain 3.

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Role of Caspases in Bax Activation and Anoikis

Cycloheximide (Calbiochem catalog no. 239764); 100 μM 19918

The concentrations of z-VAD-fmk and z-IETD-fmk are effective for quiescence of Bax within the cytosol, thereby preventing its activation and translocation to mitochondria (16). Thus, in some cells, adhesion appears to regulate the cellular decision to enter apoptosis by controlling events at the mitochondrion.

By contrast, several publications have suggested that death receptors may be involved in anoikis, thereby implicating mitochondrial-independent caspase activation. In certain epithelial cell lines, loss of integrin-mediated adhesion to the ECM results in rapid activation of caspase-8, an initiator caspase that is normally associated with the ligation of death receptors (17, 18). This leads to direct activation of effector caspases as well as cleavage of the BH3-only protein Bid. The role of caspases in detachment-induced cytochrome c mobilization, at least in MDCK and MCF-10A cells, is supported by the observation that both a dominant-negative death domain that blocks caspase-8 recruitment to the death-inducing signaling complex and a generic caspase inhibitor, z-VAD-fmk, prevent cytochrome c release (19). Furthermore, in endothelial cells, the FasL/Fas/caspase-8 axis is sensitized by detachment of cells from the ECM, although this occurs over a substantial (12-h) time frame (20).

We previously demonstrated an essential role for Bax activation in detachment-induced apoptosis of mammary epithelial cells (16). In view of the possibility that caspase-8 activation and Bid cleavage may be involved in anoikis of some epithelial cell lines, we have now addressed the question of whether or not removing integrin-mediated adhesion signals, initiator caspases are required for Bax translocation to mitochondria, cytochrome c release, and effector caspase activation.

EXPERIMENTAL PROCEDURES

Cell Culture—FSK-7 is a mouse mammary epithelial cell line isolated from luminal epithelial cells (21). FSK-7 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (BioWhittaker) supplemented with 5% epidermal growth factor, 880 nM insulin, and 2% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO2. MDCK dog kidney epithelial cells were cultured similarly; we used one strain of MDCK cells already existing within our laboratory as well as early passage MDCK-II cells purchased from the European Collection of Cell Cultures and used within three further passages. In all experiments, adherent cell monolayers were harvested after growth on tissue culture plastic or glass coverslips for 48 h, or cells were detached from serum-containing medium and plated in whole medium onto dishes with poly-HEMA for different times, washed once with PBS, and permeabilized in 100 μM caspase-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; catalog no. sc-1224), Erk1 (Santa Cruz Biotechnology catalog no. sc-154), and Bid (R & D Systems catalog no. AP560). Detection was achieved with peroxidasecoupled sheep anti-mouse or donkey anti-goat IgG (Jackson) followed by enhanced chemiluminescence (Amersham Biosciences).

Immunofluorescence—Cells were fixed with 3.7% paraformaldehyde in PBS for 10 min, cytoskeleton stained with poly-L-lysine slides (BDH), and permeabilized in 0.1% Triton X-100 in PBS before use. For rapid preparation of cytosolic fractions, cells were washed twice with PBS and resuspended in 100 μl of mitochondrial buffer (70 mM Tris-Cl, 0.25 μM sucrose, 1 mM EDTA, pH 7.4), and an equal volume of digitonin (0.2 mg/ml dissolved in MES buffer: 19.8 mM EDTA, 0.25 mM n-mannitol and 19.8 mM MES, pH 7.4) was added to the sample for 5 min (23). After centrifugation at 900 × g for 2 min, the supernatant was centrifuged further at 20,000 × g for 5 min to obtain the cytosolic fraction, which was prepared for immunoblotting. For treatment of cell lysates with caspase-8, FSK-7 cells were lysed directly in caspase digestion buffer (50 mM HEPES-Cl, 50 mM NaCl, 0.17 mM CHAPS, 10 mM EDTA, 10 mM dithiothreitol, 5% glycerol, pH 7.5), and then 150 μg of total cell protein was incubated with 1 unit of activated recombinant caspase-8 (Calbiochem catalog no. 218812) before analysis by immunoblotting.

Flow Cytometry—Cells cultured on poly-HEMA were harvested and fixed with 2% paraformaldehyde for 10 min and more, washed three times in PBS, and permeabilized in 0.5% Triton X-100 for 15 min. Cells were incubated for 1 h with 62% anti-Bax antibody at 4 μg/ml in PBS containing 0.5% Triton X-100 and 0.1% horse serum. After three washes in PBS, the cells were incubated for 30 min with 1:500 dilution fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson catalog no. 41624). Analysis was performed on a FACSVantage flow cytometer. Fluorescein isothiocyanate fluorescence was detected at 530 ± 30 nm, and fluorescence was acquired using logarithmic amplifiers. 10,000 cells were analyzed per sample at a flow rate of 300 cells/s.

RESULTS

Bax Is Activated within 15 Min of Detachment from ECM—In mammary epithelial cells, integrin function suppresses the activation of Bax (16). Upon cell detachment from ECM, Bax translocates to mitochondria. Concomitant with this, Bax undergoes a conformational change, which can be visualized using antibodies to cryptic epitopes within Bax that only become exposed following its activation. To determine whether Bax...
activation, as measured by a change in conformation, is an early initiator of the apoptotic program in response to cell detachment, we examined the kinetics of events following the removal of mammary epithelial cells from ECM. After plating onto the nonadhesive substrate, poly-HEMA, cells were cytospun at various time intervals. Bax activation was examined by immunostaining with a polyclonal anti-peptide antibody that has previously been shown to recognize only the activated form of Bax on a specific epitope overlapping the BH3 domain, 62M (22). As in adherent cells, there was little or no exposure of the 62M epitope within 5 min following detachment from ECM (Fig. 1a). However, Bax became immunoreactive 10–15 min after loss of adhesion and was evident in the majority of the cells. Exposure of the Bax 62M epitope was maintained throughout 24-h culture on poly-HEMA and during the morphological changes associated with apoptosis.

**Fig. 1. Kinetics of Bax activation following loss of integrin-mediated adhesion.**

- **a**, adherent FSK-7 cells and FSK-7 cells maintained on poly-HEMA for the indicated times prior to fixation and centrifugation onto polysine slides were immunostained with an antibody that recognizes an activation state epitope within Bax, 62M, and nuclei were counterstained with Hoechst 33258. Note the absence of Bax immunoreactivity in attached cells and those detached for 5 min and that staining becomes apparent 10–15 min after detachment and is maintained thereafter. All of the Bax immunoreactivity could be abolished by preincubation with a Bax peptide corresponding to the immunogen. 
- **b**, cells detached for the indicated times were immunostained with the anti-Bax 62M antibody and analyzed by flow cytometry. Note the rapid increase of fluorescence over the first 10–15 min after loss of integrin-mediated adhesion, which reaches a maximum after 15 min in suspension. 
- **c**, adherent FSK-7 cells and FSK-7 cells maintained on poly-HEMA for the times indicated prior to hypotonic extraction and Dounce homogenization were subfractionated by centrifugation at 100,000 × g into a soluble (S) fraction and a pellet (P) fraction. Equal fractions of protein were resolved by SDS-PAGE, and immunoblots were probed with anti-Bax 62M and anti-actin antibodies. 
- **d**, FSK-7 cells were transfected with a plasmid expressing Bax conjugated to YFP (YFP-Bax) and either left attached to the culture dish or detached from the dish and incubated on poly-HEMA for 15 min. The cells were fixed, stained with an antibody to mitochondrial HSP70 (mtHSP70) to identify mitochondria, and visualized by immunofluorescence microscopy. Note the even staining of YFP-Bax through the cytosol, virtually all of which costains with mitochondrial HSP70 after cell detachment from extracellular matrix.
To quantify Bax activation, detached cells were fixed at the same time points after incubation on poly-HEMA, and stained with the 62M antibody for FACS analysis (Fig. 1b). There was little Bax staining immediately after detachment, but this rapidly increased over 10–15 min. Exposure of the 62M epitope was maximal after 15 min in suspension and remained at this level over several hours. At later time points, Bax staining became slightly reduced, although this is probably due to cells undergoing the terminal stages of apoptosis. We did find, however, that cells containing apoptotic bodies still stain for active Bax.

Two experiments were performed to confirm that Bax translocated to mitochondria. First, mammary cells were extracted under detergent-free, hypotonic conditions, and the soluble and membrane fractions were separated. Under these conditions, Bax was largely present in the cytosol of adherent cells (Fig. 1c). Following detachment from the ECM, the majority of Bax was associated with the membrane fraction. This occurred within 15 min of detachment and correlated exactly with the kinetics of exposure of the 62M epitope of Bax. Second, cells were transfected with YFP-Bax, and its location was examined in adherent cells and in those detached from the substratum. YFP-Bax was present in a diffuse, cytosolic distribution in adherent cells (Fig. 1d). Following cell detachment from the ECM for 15 min, YFP-Bax became punctate and co-stained with the mitochondrial marker mitochondrial HSP70, indicating its redistribution to mitochondria.

These data suggest that following cell detachment, Bax ac-
tivation and its translocation to mitochondria is a very rapid event and is complete within 15 min of removal of integrin signaling. This appears to occur synchronously within the cell population.

A Delay Occurs between Bax Activation and the Release of Cytochrome c from Mitochondria—The rapidity of Bax activation is in contrast to mammary epithelial cell death, which occurs over several hours following loss of adhesion. To compare Bax activation with events previously indicated to characterize commitment to apoptosis, we examined the kinetics of cytochrome c release from mitochondria and the activation of caspase-3.

Cells maintained on poly-HEMA were extracted with digitonin to leave mitochondria intact, and the soluble extracts were immunoblotted for cytochrome c. Although the Bax 62M epitope became exposed within 15 min of cell detachment from ECM, cytochrome c was not released at this time. Rather, the appearance of cytochrome c in the cytosol was first apparent 1 h following detachment and subsequently increased to a maximal level at 2 h (Fig. 2a).

Cytochrome c release from mitochondria has been described to occur rapidly and, once initiated, to be complete within 5 min subsequent to the treatment of cells with a variety of apoptotic stimuli (24). This observation is not consistent with the gradual appearance of cytochrome c in the cytosol over a period of hours in ECM-detached mammary cells. To determine whether cytochrome c is released from mitochondria in a rapid and kinetically invariant manner following loss of integrin survival signals, we examined its subcellular distribution within individual cells in situ. After 1 h, when cytochrome c is first detectable in the cytosol by immunoblotting, most cells still showed it localizing to the mitochondria by immunofluorescence (Fig. 2b). At later time points, the percentage of cells without mitochondrial cytochrome c staining increased, and many of the cells displayed diffuse cytochrome c staining instead (Fig. 2, b (arrows) and c). These results suggest that the slow release of cytochrome c observed in immunoblotting is due to its release in an asynchronous manner. Importantly, the rapid and synchronous conformational changes that occur within Bax following loss of adhesion do not lead immediately to the release of cytochrome c from mitochondria.

Cytochrome c release into the cytosol is required for the formation of the apoptosome, within which the proenzymatic form of caspases are cleaved to generate the active enzyme (25). This example, in sympathetic neurons, the removal of neurotrophins rapidly commits cells to caspase activation and apoptosis (26). We therefore examined the kinetics of caspase-3 activation in response to cell detachment and compared this with the temporal release of cytochrome c. Activation of the effector caspase, caspase-3, was monitored in cells cultured on poly-HEMA by examining the appearance of its cleaved active subunit. Active caspase-3 was just detectable 2 h after detach into mito mammmary cells from ECM and was significantly more pronounced at 8 h, as measured both by immunoblotting (Fig. 2a) and immunofluorescence (Fig. 2b). This time course was confirmed using a fluorometric assay to measure caspase-3 enzyme activity (data not shown). We also noticed that some of the cells with cytochrome c released from mitochondria contained nuclei with normal morphology (Fig. 2b, arrowheads at the 1- and 8-h time points). Rather than becoming apoptotic just after cytochrome c release, the number of cells with normal nuclei gradually disappeared over time as the proportion of cells without mitochondrial cytochrome c but containing apoptotic nuclei increased (Fig. 2c). Thus, in anoikis, there is a lag between the release of cytochrome c and the activation of caspases, leading to the subsequent morphological signs of apoptosis.

Taken together, these results define a sequential ordering of apoptotic events following cell detachment from the ECM. In mammary cells that adhere to the ECM via integrins, Bax is localized within the cytosol and excluded from mitochondria. 15 min after cell detachment, Bax is redistributed to mitochondria and undergoes conformational changes to reveal the 62M epitope. These changes occur synchronously within the population of detached cells. Over the next 1–8 h, cytochrome c is released asynchronously into the cytosol, possibly in a stochastic manner. This is followed by the activation of caspase-3 and subsequently by cell death. Importantly, our results firmly place the activation of Bax and its translocation to mitochondria as one of the earliest detectable events leading to apoptosis after loss of integrin signaling.

Anoikis in Mammary Cells Does Not Require de Novo Protein Synthesis—Although the ECM is a survival factor for mammary cells, other types of extracellular ligand can also regulate survival through Bax translocation in some cell systems. For example, in sympathetic neurons, the removal of neurotrophins results in Bax translocation to mitochondria and apoptosis in a...
We examined whether this was the case by detaching cells in the presence of cycloheximide and measuring the kinetics of Bax activation, cytochrome c release from mitochondria, and apoptosis. Cycloheximide did not delay the kinetics of Bax activation as measured by exposure of the Bax 62M epitope in both immunofluorescence and FACS analysis (Fig. 3a). Similarly, cytochrome c release still occurred when protein synthesis was blocked. ImmunobLOTS of isolated cytosolic fractions show that cytochrome c was released after 1 h, with caspase-3 activation after 2–4 h (Fig. 3b). This time course compares with that in the absence of cycloheximide (Fig. 2a). Finally, there was no difference in the rate of apoptosis over a 24-h time course in detached cells treated with or without cycloheximide, indicating that apoptosis occurs equally in the presence or absence of protein synthesis (Fig. 3c).

These data indicate that despite the delay between Bax translocation and cytochrome c release and caspase-3 activation, the anoikis program in mammary epithelial cells is independent of protein synthesis. Moreover, the mechanism for Bax redistribution and the subsequent events in the progression of apoptosis vary in response to the removal of different types of survival factor (i.e. trophic factors from sympathetic neurons and ECM from mammary epithelial cells).

**Premitochondrial Caspase Activation Is Not Involved in Anoikis**—It has been reported that anoikis may be initiated by death receptor-mediated activation of caspase-8 (17, 18, 20). Our results have indicated that Bax is redistributed rapidly to mitochondria in adhesion-regulated apoptosis (Fig. 1). Since caspase-8 can cleave the BH3-only protein Bid and it has been suggested that cleaved tBid may have a role in the activation of Bax, there is a possibility that Bax may be activated indirectly following caspase-8 activation (28–32). We therefore examined the possibility that caspases, in particular the death receptor-activated caspase-8 (which is expressed in mammary epithelial cells) (33), might play a role in mammary cell anoikis.

We first compared the effects of a broad spectrum inhibitor of caspase activation, z-VAD-fmk, with a specific caspase-8 inhibitor, IETD-CHO, on Bax activation. Both of these inhibitors prevent the activation of caspase-8 in cells by staurosporine (Fig. 4d). Neither z-VAD-fmk nor IETD-CHO blocked exposure of the Bax 62M epitope, when examined by immunostaining or FACS analysis (Fig. 4a, a and b). Furthermore, the kinetics of Bax conformation changes were not altered following caspase inhibition, with maximal activation occurring 15 min after cell detachment from ECM.

To determine whether caspase-8 is required for cytochrome c release, we examined its appearance in the cytosol in the presence or absence of z-VAD-fmk or IETD-CHO. Neither of these caspase inhibitors delayed the release of cytochrome c from mitochondria (Fig. 4c). Together, the results imply that caspase-8 is not required for detachment-induced Bax activation and cytochrome c release.

Death receptors are present on mammary cells (34, 35). Fas ligand, tumor necrosis factor-α, and TRAIL all induced apoptosis in mammary epithelial cell cultures, indicating that the death receptor axis can be activated, provided the appropriate signals are present. To confirm the lack of involvement of death receptor-activated caspases in mammary epithelial anoikis, we examined the cleavage of one of its substrates, Bid, following detachment from ECM. Although treatment of mammary cell lysates with purified caspase-8 resulted in the appearance of cleaved tBid, which was blocked by z-VAD-fmk and IETD-CHO (Fig. 5, a and b), staurosporine induced the disappearance of full-length Bid in MDCK epithelial cells (data...
not shown), we could not find any evidence for Bid cleavage in mammary cells plated in suspension on poly-HEMA (Fig. 5c).

Since caspases are not required for Bax activation or cytochrome c release (Fig. 4), we investigated whether the caspase inhibitors had any effect on later apoptotic events. Although z-VAD-fmk greatly delayed caspase-3 activation, IETD-CHO had no appreciable effect (Fig. 6a). In agreement with this result, only z-VAD-fmk, but not IETD-CHO, delayed cell death in suspension (Fig. 6b). As a control for these experiments, to demonstrate that Bax activation can be inhibited by pharmacological agents following the loss of adhesion to ECM, detached cells were also treated with the protein-tyrosine phosphatase inhibitor, vanadate (Fig. 7). This efficiently blocks both Bax activation and apoptosis.

Together, our results demonstrate that premitochondrial caspases are not required to mediate the initial events that lead to cytochrome c release and apoptosis. Instead, a rapid death receptor-independent signal mediated by loss of integrin ligation results in Bax activation.

**DISCUSSION**

All mammalian cells require the receipt of extracellular signals to survive. In many cell types, interactions with adhesive ligands, in addition to soluble factors, are essential for the prevention of apoptosis. An implication of this is that cell survival in vivo is mediated not only by the milieu of locally acting factors but also by spatial cues within the cell and tissue microenvironment. Adhesive interactions with the ECM can occur through integrins, and there is now abundant evidence that these receptors provide essential survival signals in vivo (36–38). However, the intracellular signaling mechanisms by which integrins suppress apoptosis are varied and have not yet been explored fully.

We have previously demonstrated that apoptosis resulting from loss of integrin-mediated adhesion in mammary epithelial cells occurs through translocation of Bax from the cytosol to mitochondria (16). Bax activation, as revealed by conformational changes that lead to exposure of the 62M antibody epitope, occurs after its translocation to mitochondria.3 Here we have confirmed that Bax structure alters after mammary

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3 A. J. Valentijn and A. P. Gilmore, unpublished data.
epithelial cells are detached from the ECM but demonstrate that it occurs far more rapidly than previously thought, within 15 min of loss of integrin signaling; at this time, the majority of Bax has translocated from the cytosol to membrane as determined by subcellular fractionation studies. Moreover, the changes are synchronous within the cell population, since all cells show conformationally altered Bax by immunofluorescence and FACS analysis.

Although the initial integrin survival signal appears to be mediated through focal adhesion kinase, it is not yet known what the downstream signals are that lead to changes in the conformation of Bax. One possible mechanism might involve activation of the extrinsic pathway for apoptosis, which has been implicated in several cell systems where apoptosis is triggered following altered integrin signaling. This pathway is normally activated in response to ligation of death receptors by their ligands, an event that involves formation of the death-inducing signaling complex and activation of caspase-8 and thereby both the direct activation of effector caspases and their indirect activation via Bid and the mitochondrial route (1). Bid is a substrate for caspase-8, whose product, tBid, translocates to mitochondria and ultimately leads to cytochrome c release (39). In some situations, Bid is essential for the extrinsic apoptosis pathway, since an activating Fas antibody does not kill hepatocytes from Bid-null mice (40).

In some strains of MDCK cells, detachment from ECM induces caspase-dependent cytochrome c release from mitochondria, and specific inhibitors of caspase-8 block both cytochrome c release and apoptosis in MDCK and endothelial cells (19, 20). In another cell culture model, artificial overexpression of integrins in adherent carcinoma cells leads to recruitment and activation of caspase-8, thereby triggering apoptosis (41). These mechanisms for apoptosis induction are distinct, since the former appears to involve death receptor adaptors, whereas the latter entails the direct recruitment of caspase-8 to integrin tails.

Other types of cells, such as mammary epithelial cells, are also dependent on integrins for their survival, and we have therefore asked whether anoikis in this system involves activation of caspase-8. However, our data indicate that this is not the case, since the early events of the anoikis program in mammary cells are independent of caspase activation. The evidence for this is that conformational changes within Bax occur very rapidly after the inhibition of integrin signals, with kinetics that are not altered by either a caspase-8 or a generic caspase inhibitor. Moreover, the subsequent release of cytochrome c is also not dependent on caspase activation in mammary cells. This is supported by our study, where Bid cleavage is not apparent following loss of adhesion of mammary cells.

Thus, our data demonstrate that anoikis does not always require the activation of initiator caspases but can in certain cell types be dependent on the mitochondrial apoptosis program. Interestingly, we have found that in some strains of MDCK cells, anoikis is also not dependent on caspase-8 activation (data not shown). Caspase-independent changes in integrin signaling lead to rapid changes in Bax, and we are currently identifying the pathway of enzymes involved in mammary epithelial cells. Possibilities include the phosphatidylinositol 3-kinase-protein kinase B pathway, which has a role in staurosporine-induced Bax translocation in HeLa cells, or c-Jun N-terminal kinase, since a constitutively active version mediates apoptosis via Bax in embryonic fibroblasts and Chinese hamster ovary cells (15, 42). One model for Bax activation invokes the involvement of upstream BH3-only proteins, and Bim is a possible candidate since certain splice variants of this protein influence the conformation and activation of Bax (43). Whichever pathway is involved, it is likely to be direct rather than via a transcriptional intermediate. In neuronal cells, Bax activation and apoptosis following nerve growth factor withdrawal are dependent on RNA and protein synthesis (27, 44). This is not the case in mammary cells, since cycloheximide has no effect on either the kinetics of Bax activation or apoptosis following loss of integrin signaling.

Once Bax has become conformationally altered after the removal of adhesion survival signals, the ultimate consequence is a change in mitochondrial structure that precedes export of cytochrome c (and other proapoptotic proteins) across the outer mitochondrial membrane and thus activation of executioner caspases. In some cell systems, activated BH3-only proteins trigger this process efficiently and rapidly. tBid remodels the structure of isolated liver mitochondria and induces cytochrome c release in permeabilized HepG2 cells within 10 s (39, 45). Although cytochrome c export can happen rapidly once it has been triggered and occurs synchronously from all mitochondria within individual cells (as judged by kinetic analysis of green fluorescent protein-cytochrome c movements), not all cells release cytochrome c at the same time (24). UV-treated HeLa cells release cytochrome c stochastically over a period of 5 h, beginning 3 h after the death stimulus. Similarly, we find that cytochrome c is not released from all mammary epithelial cells simultaneously after removing integrin survival signals. Rather, it occurs stochastically, beginning ~2 h after detachment from ECM.

![Image](https://example.com/image.png)
The changes within mitochondrial structure and the precise mechanisms for release of cytochrome c are not known and currently are a subject of considerable debate (46). However, the significant lag of more than 2 h between Bax conformational change in mammalian cells and observable cytochrome c release indicates that the changes are profound and complex and suggest that apoptosis commitment events may require other cellular decisions. Our culture model may provide an excellent opportunity for experimental dissection of the apoptotic decision events that occur following the initial activation of Bax. Moreover, it may help to resolve the question of why the initial, synchronous changes in Bax conformation lead only to a nonsynchronous, possibly stochastic release of cytochrome c from mitochondria.

In summary, this paper reports two significant and novel findings about the mechanism of integrin-regulated survival. The first is that loss of adhesion of mammalian epithelial cells to ECM results in the activation of an intrinsic apoptosis pathway, which does not involve caspase-8. The second is that altered integrin signals induce rapid and synchronous changes in the conformation of Bax, but the apoptosis commitment event leading to cytochrome c release and caspase activation occurs asynchronously within the cell population and after a time delay.

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