Bax activation and translocation to mitochondria mediate EGF-induced programmed cell death

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Summary

The ErbB family of receptor tyrosine kinases is involved in the regulation of cell proliferation, differentiation and apoptosis. Previous studies indicate that cells expressing elevated levels of the EGFR and ErbB-2 undergo programmed cell death in response to EGF or other EGFR ligands. However, the detailed mechanisms of EGF-induced apoptosis are unclear. This report demonstrates that in the cells undergoing EGF-dependent apoptosis Bax changes its conformation and forms multimeric aggregates, which accumulate on the mitochondrial membrane. Bax activation and translocation to the mitochondria induces a loss of mitochondrial transmembrane potential and cell death. Also, during EGF-induced apoptosis there is downregulation of Bcl-xL, an anti-apoptotic protein. Expression of Bcl-xL in cells susceptible to EGF-dependent apoptosis prevents cell death. The data indicate that addition of EGF does not result in a significant release of cytochrome c from mitochondria and EGF-induced apoptosis is mainly caspase independent.

Key words: EGFR, ErbB-2, Bax, Apoptosis, Mitochondria

Introduction

The EGF receptor (or ErbB) family includes four type I receptor tyrosine kinases, which are expressed in a wide variety of tissues, and plays a pivotal role in the proliferation and differentiation of cells of epithelial and other origins (Prenzel et al., 2001; Yarden and Sliwkowski, 2001). Ligand-induced dimerization of the EGF receptor (EGFR) results in activation of its kinase and stimulation of various signaling pathways. ErbB-2 is a preferred heterodimerization partner with all other ErbB receptors and a potent activator of pro-survival signaling pathways. It has no known ligand and can be activated through heterodimerization with EGFR or other members of the ErbB family (Hynes et al., 2001; Klapper et al., 2000).

While EGF and ErbB-2 usually mediate cell proliferation in vivo and in cell culture, it has also been reported that both receptors may stimulate cell differentiation or activate an apoptotic program (Casalini et al., 2004; Danielsen and Maihle, 2002). EGF induces apoptosis in A431 or MDA-468 cells that express a high number of EGFRs (Filmus et al., 1985; Gill and Lazar, 1981). It has been suggested that EGF-induced apoptosis in A431 cells requires STAT1 activation followed by induction of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 (Chin et al., 1996). While it was proposed that EGFR activation stimulates expression of caspase 1 (Chin et al., 1997), recent work also suggests that EGF-induced apoptosis may proceed through a caspase-independent pathway (Fombonne et al., 2004).

Apoptosis, or programmed cells death, is a genetically determined process regulating the development and tissue homeostasis of multicellular organisms. There are two major evolutionarily conservative pathways of apoptosis: activation of cysteine-aspartic proteases known as caspases and mitochondrial outer membrane permeabilization (Green, 2000; Strasser et al., 1995). Each pathway is dispensable as apoptosis may proceed without caspase activation or in cells devoid of mitochondria (Bratosin et al., 2001). However, these pathways usually operate together to accelerate cell death.

The caspase-dependent pathway involves the proteolytic activation of a family of cysteine proteinases that cleave substrates after a conserved aspartate residue. Ligand binding to TNF receptor, CD95, or other proteins from the ‘death receptors’ superfamily stimulate the recruitment of several adaptor proteins and specific pro-caspases, such as pro-caspase 8 and 10, into protein complex known as the death-inducing signaling complex (DISC). Once activated, apical caspases activate downstream executioner caspases 3, 6, 7 resulting in the amplification of the caspase cascade, cleavage of many critical cellular protein substrates and induction of DNA fragmentation (Nicholson and Thornberry, 1997; Villa et al., 1997).

Heat shock, oxidative stress, or DNA damage initiate an intrinsic apoptotic pathway intimately related to mitochondrial membrane integrity. These stimuli induce a loss of the mitochondrial membrane potential (MMP), which results in release of several apoptotic regulatory proteins, including cytochrome c, pro-caspase 9, and sometimes several other pro-caspases (2, 3, 12), depending on the cell type, into the cytosol. Released cytochrome c binds to the activator APAF-1 and recruits caspase 9 into a complex known as the apoptosome. Apoptosome-activated effector caspases then trigger downstream events leading to apoptosis (Brenner et al., 1998; Earnshaw, 1999).

The loss of MMP is regulated by the Bcl-2 family proteins. Two closely related proteins from the Bcl-2 family, Bax and...
Bak, whose expression levels vary in different tissues, have overlapping roles in the regulation of apoptosis (Lindsten et al., 2000). In the absence of both proteins, the apoptotic program based on mitochondrial dysfunction in response to different stimuli is severely impaired (Wei et al., 2001). Bak normally resides in the cytosol, whereas Bak is located on the mitochondrial membrane. Apoptogenic signals promote conformational changes of Bak and Bak, which in some cellular models are dependent on an interaction with proteolytically cleaved Bid, a pro-apoptotic member of the Bcl-2 family (Desagher et al., 1999; Wei et al., 2000). Conformational changes induce oligomerization of Bak and Bak and allow Bak mitochondrial docking (Nechushtan et al., 1999). Both Bak and Bak induce the formation of pores in the outer membrane, resulting in a loss of the MMP followed by cell death (Kuwana et al., 2002).

Mitochondrial intermembrane space contains factors, such as apoptosis-inducing factor (AIF) and endonuclease G, which trigger DNA fragmentation and chromatin condensation. In response to apoptotic stimuli both factors can be released from mitochondria and translocated to the cytosol and the nucleus. Addition of these factors to the purified nuclei results in apoptotic changes even in the presence of caspase inhibitors, leading to the conclusion that activation of AIF and endonuclease G represents a novel caspase-independent apoptotic pathway (Penninger and Kroemer, 2003). Recent studies suggest that although AIF and endonuclease G induce DNA fragmentation in a caspase-independent fashion, their translocation depends on caspase activation (Arnoult et al., 2003).

It has been proposed that the cellular outcome of signaling through the EGFR depends on the level of EGFR expressed in cells (Hoganson et al., 2001). We have found that EGFR activation stimulates cellular proliferation in cells with low to moderate levels of the receptor, while in cells that overexpress EGFR, activation of the receptor leads to apoptosis. ErbB-2 co-expression and heterodimerization with the EGFR strongly potentiates apoptotic signaling through both receptors in a variety of cell lines (Tikhomirov and Carpenter, 2004). The molecular mechanism of apoptosis induced by EGF in these cells is unclear. Here we show that binding of EGF to its receptor in cells with elevated levels of EGFR and ErbB-2 results in activation of Bax and its docking to the mitochondrial membrane. Bak translocation leads to the loss of mitochondrial membrane potential and apoptotic cell death. An important feature of EGF-induced programmed cell death is its independence from caspase activation, which makes it distinct from the ‘classical’, caspase-dependent apoptosis. Both caspase-dependent and -independent types of programmed cell death have similar morphological manifestations of apoptosis and historically the term ‘apoptosis’ was applied to the type of programmed cell death that involves a loss of MMP. Therefore, we also use it to describe EGF-induced programmed cell death.

**Materials and Methods**

**Materials**

A cDNA encoding EGFR fused to GFP (EGFP-C1 vector) was a generous gift from Alexander Sorkin (University of Colorado Health Science Center), while the ErbB2-RFP/pDNA3.1hygro vector was described elsewhere (Tikhomirov and Carpenter, 2004). The EGFP vector containing pDsRed2-Mito vector and the pIRES2-EGFP were purchased from BD Biosciences. The Bax cDNA was a gift from Elizabeth Yang and Bruce Carter. Goat anti-mouse antibody crosslinked with horseradish peroxidase was from Zymed. Goat anti-mouse antibody crosslinked with Alexa 488 or Alexa 546 and MitoTracker CMTMRos were from Molecular Probes. 6A7 antibody was obtained from Sigma, anti-cytchrome c antibody was from Santa Cruz, while antibodies against Bak, Bcl-xL and phosphotyrosine were from BD Biosciences. LipofectAMINE was purchased from Gibco-BRL. Caspase inhibitors and staurosporine were obtained from Calbiochem.

**Cell lines and cell culture**

MCF7 cells stably expressing EGFR*GFP and ErbB-2*RFP were transfected with EGFR*GFP/pEGFP-N1 and ErbB-2*RFP/pDNA3.1hygro vectors and stable cell lines were selected in the presence of G418 and hygromycin. Clones with high levels of EGFR and ErbB2, as confirmed by fluorescent microscopy and western blotting, were used. Cells were grown in DMEM containing 10% FBS to 60-80% confluency and then treated with EGF or vehicle for 4-5 days.

A431/ErbB2 cells were transfected with GFP*Bax/pEGFP-C1, Bcl-xL/pIRES2-EGFP, or pDsRed2-Mito constructs and stable cell lines expressing Bax, Bcl-xL or RFP-mito were selected by flow cytometry using GFP or RFP as markers. The A431/ErbB2/Bax cells were transfected with pDsRed2-Mito vector to select a cell line stably expressing GFP*Bax and RFP targeted to mitochondria. The cells were grown in DMEM containing 10% FBS and viability was assessed by Trypan Blue exclusion.

**Construction of vectors**

The Bax expression construct was prepared as a GFP fusion protein. The PCR product was amplified using primers ACA TCT CGA GCT CAA ATG GAC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech) through SacI and SalI restriction sites. Bcl-xL cDNA was prepared by RT-PCR from total RNA isolated from SKBr3 cells using primers ATT ACT CCT CGA GCT GAC CCC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech) through SacI and SalI restriction sites. Bcl-xL cDNA was prepared by RT-PCR from total RNA isolated from SKBr3 cells using primers ATT ACT CCT CGA GCT GAC CCC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech). Bcl-xL cDNA was prepared by RT-PCR from total RNA isolated from SKBr3 cells using primers ATT ACT CCT CGA GCT GAC CCC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech) through SacI and SalI restriction sites. Bcl-xL cDNA was prepared by RT-PCR from total RNA isolated from SKBr3 cells using primers ATT ACT CCT CGA GCT GAC CCC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech) through SacI and SalI restriction sites. Bcl-xL cDNA was prepared by RT-PCR from total RNA isolated from SKBr3 cells using primers ATT ACT CCT CGA GCT GAC CCC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech) through SacI and SalI restriction sites. Bcl-xL cDNA was prepared by RT-PCR from total RNA isolated from SKBr3 cells using primers ATT ACT CCT CGA GCT GAC CCC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech) through SacI and SalI restriction sites. Bcl-xL cDNA was prepared by RT-PCR from total RNA isolated from SKBr3 cells using primers ATT ACT CCT CGA GCT GAC CCC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech). Bcl-xL cDNA was prepared by RT-PCR from total RNA isolated from SKBr3 cells using primers ATT ACT CCT CGA GCT GAC CCC GGG TCC GGG GAG C and ACA TGA GTC GAC TCA GCC CAT CTT CCT CCA GAT GGT, and was inserted into pEGFP-C1 vector (Clontech).

**Confocal microscopy**

Confocal images were captured on a LSM510 microscope with a Plan-Neofluar 40× 1.3 NA or 100× 1.3 NA objective (Zeiss). The cells were grown on 35 mm MatTek glass-bottom microwell dishes (glass thickness 1.5), then treated as described in the figure legends. To detect the loss of the mitochondrial potential, the cells were incubated for 30 minutes with MitoTracker CMTMRos dye, which is sequestered by actively functioning mitochondria, washed twice with medium and fixed with 4% paraformaldehyde.

For antibody staining the cells were treated with EGF or vehicle for the indicated times and fixed for at least 30 minutes with 4% paraformaldehyde. Next, the cells were permeabilized with 0.4% Triton X-100 for 15 minutes and incubated for 2 hours or overnight in blocking solution (2% BSA in PBS). For cytchrome c and 6A7 staining, the cells were incubated overnight at 4°C with the corresponding antibodies, in blocking solution. Staining with other antibodies was performed for 2 hours at room temperature. After staining with primary antibody, the cells were washed three times for 15 minutes with PBS and incubated for 1 hour with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 546. The cells were washed again three times, and then rinsed with distilled
water. The dishes were dried and glass bottoms were removed and attached to glass slides using Aqua PolyMount.

Cell fractionation

Cells were scraped into 10 ml of ice-cold PBS, collected by centrifugation and washed twice with PBS. The cells were resuspended in 1 ml of 1X cytosol extraction buffer of the Cytosol/Mitochondria Fractionation Kit (Oncogen/Calbiochem), incubated 10 minutes on ice, homogenized and centrifuged at 700 g for 10 minutes. The supernatant was centrifuged again at 10,000 g for 30 minutes. The resulting supernatant was the cytosolic fraction, and the pellet was the mitochondrial fraction.

Immunoblotting

At the end of each experiment the cells were solubilized by scraping into cold lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM Na3VO4) or CHAPS buffer (Cell Signaling Technologies) for caspase detection. The lysates were then clarified by centrifugation (14,000 g, for 10 minutes) mixed with Laemmli sample buffer and boiled. Cytosol/mitochondrial fractions were directly lysed in 1X Laemmli sample buffer. Aliquots containing equal amounts of protein were subjected to SDS-PAGE. Subsequently, proteins were transferred to nitrocellulose membranes and the membrane was blocked by incubation with 5% BSA in PBS for 1 hour at room temperature. The membrane was then incubated for 1 hour (overnight at 4°C for antibodies against caspases and 6A7 antibody) with the indicated antibody in TBSTw buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.2% nonfat milk), washed three times in the same buffer and incubated for 1 hour with HRP-conjugated secondary antibody or protein A. The membrane was then washed five times with TBSTw buffer and visualized by enhanced chemiluminescence (ECL).

Results

Previous studies showed that EGF consistently induces apoptosis in cells that express high levels of EGFR and that the rate of cell death is significantly increased if EGFR is co-expressed with ErbB-2. Parental SKBr3 breast carcinoma cells that have low levels of EGFR and high levels of ErbB-2 do not undergo apoptosis in the presence of EGF. However, SKBr3/EGFR cells, which express higher levels of EGFR, die after 4-5 days treatment with EGF. Similar to this, expression of ErbB-2 in A431 cells, which have a high endogenous level of EGFR, accelerates apoptosis (Tikhomirov and Carpenter, 2004).

Caspase activation does not play a significant role in EGF-dependent cell death

Caspase activation usually plays an important role in committing a cell to apoptotic death in response to many drugs or after the engagement of cell death receptors. Previously, we have reported that caspase 3 activation was not detected in SKBr3/EGFR and A431/ErbB-2 cells treated with EGF (Tikhomirov and Carpenter, 2004). However, additional experiments using different antibodies against activated caspases show that there is some activation of caspases 3 and 7 in both SKBr3/EGFR and A431/ErbB-2 cell lines (Fig. 1A).

Since it is known that MCF7 breast carcinoma cells are deficient in caspase 3, we co-transfected MCF7 cells with EGFR and ErbB-2 and selected stable cell lines that express elevated levels of both receptors. Parental MCF 7 cells do not have significant levels of EGFR or ErbB-2 and the addition of EGF stimulates neither proliferation nor apoptosis. In contrast, MCF7/EGFR/ErbB-2 doubly transfected cells undergo apoptosis in the presence of EGF, even though they are devoid of a major effector caspase (Fig. 1B). Activation of caspases 3

Fig. 1. Limited role of caspase 3 and 7 activation in EGF-induced cell death. (A) A431/ErbB2 or SKBr3/EGFR cells were grown until 70-80% confluency and then treated with EGF (20 ng/ml) or vehicle in the presence or absence of caspase inhibitors (Z-VAD-FMK, 30 μM; caspase inhibitors 3 and 9, 20 μM) for the indicated times. Staurosporin-treated cells were used as a positive control. The cells were lysed in CHAPS buffer as indicated in Materials and Methods. The levels of activated caspases were detected by western blotting using antibodies against cleaved caspase 3 and caspase 7. (B) MCF7 cells, which are devoid of caspase 3, were transfected with EGFR*GFP and ErbB-2*RFP constructs and stable cell lines were selected. The cells were incubated with or without EGF for 4 days and images were prepared using fluorescence microscopy.
and 7 can be blocked by a pan-caspase inhibitor Z-VAD-FMK or inhibitors of the individual caspases 3 or 7, as shown in Fig. 1A. However, the same concentrations of inhibitors do not prevent EGF-induced cell death in SKBr3/EGFR, A431/ErbB-2 or other cell lines that undergo apoptosis in an EGF-dependent fashion (Tikhomirov and Carpenter, 2004). These findings confirm the conclusion that caspase activation does not play a crucial role in EGF-induced apoptosis.

Stable expression of Bax in A431/ErbB-2 cells accelerates the onset of apoptosis

Because inhibition of caspases does not block apoptosis and a loss of MMP has been found in cells undergoing apoptosis in response to EGF (Tikhomirov and Carpenter, 2004), the hypothesis that EGF-induced apoptosis and loss of MMP is dependent on Bax translocation to mitochondria was tested. To determine whether Bax intracellular levels influence apoptosis, A431/ErbB-2 cells were transfected with a construct encoding GFP fused to the Bax N terminus. Although transfection of Bax results in death in >90% of transfected cells, several clones survived and were selected. The A431/ErbB2/Bax cells grow normally with a slightly increased basal rate of apoptosis. The presence of Bax has a dramatic effect on EGF-dependent apoptosis. EGF-induced apoptosis in parental A431/ErbB-2 cells usually occurs over 3-4 days. However, expression of the Bax-GFP fusion protein significantly increases the rate of apoptosis as nearly all cells die after 24 hours and many during first 14-16 hours of incubation with EGF (Fig. 2).

EGF induces changes in Bax intracellular localization and activates Bax during apoptosis

To assess whether EGF stimulates Bax translocation we tested two cell lines, A431/ErbB-2 and SKBr3/EGFR, which undergo apoptosis in response to EGF treatment. Subcellular fractionation of the cells was performed after incubation with EGF for 1-3 days. EGF significantly increased the amount of Bax associated with the mitochondrial fraction in both A431/ErbB-2 and SKBr3/EGFR cells. In contrast, the addition of EGF to the SKBr3 cells, which express low levels of EGFR and high levels of ErbB-2 and do not exhibit apoptotic morphological changes in response to EGF, does not increase the amount of Bax in the mitochondria fraction (Fig. 3A). Bax translocation in response to EGF was confirmed by confocal microscopy. Bax expressed in A431/ErbB2/Bax cells has a cytosolic localization. In response to EGF treatment, the Bax-GFP fusion protein changes its subcellular localization and is concentrated in punctate foci (Fig. 3B). The time required for Bax translocation varied between 8 and 24 hours for individual cells.

In response to variable apoptotic stimuli Bax changes its conformation and forms oligomers. Clusters of Bax can be detected by the 6A7 antibody, which recognizes an N-terminal epitope exposed after Bax activation (Nechushtan et al., 1999). Western blotting shows a progressive increase in the amount of activated Bax in the mitochondrial fraction from SKBr3/EGFR cells treated with EGF for various times, as detected by 6A7 antibody (Fig. 4A). In contrast, there is no activation of Bax in parental SKBr3 cells. Activated Bax has also been detected in mitochondrial fractions of EGF-treated A431/ErbB2 cells (data not shown). Examination of A431/ErbB2/Bax cells by confocal microscopy shows that there is no association of 6A7 antibody with Bax in untreated cells. However, following the addition of EGF and Bax translocation into foci, activated Bax can be detected with the 6A7 antibody, indicating that Bax translocation coincides with its activation. After a 24 hours incubation with EGF most A431/ErbB2/Bax cells contain activated Bax (Fig. 4B).
Activated Bax colocalizes with mitochondria in cells treated with EGF

To further examine Bax redistribution in response to EGF, we selected an A431/ErbB2-mito cell line, which was transfected with the pDsRed2-Mito vector (Clontech) that expresses the mito-DsRed2 fusion protein targeted to the mitochondria by a sequence from subunit VIII of human cytochrome c oxidase. The cells were treated with EGF for 8–48 hours and then stained with 6A7 antibody. Untreated cells show no staining with the antibody (Fig. 5A) and even when the signal is strongly amplified, cell images show only an evenly distributed background staining. After an 8-hour incubation with EGF only a few cells exhibit weak 6A7 staining that is colocalized with mitochondria (data not shown). In contrast, after a 16–24-hour incubation with EGF in most cells antibody staining colocalizes with the mitochondrial network (Fig. 5B). It is notable, that at this time most mitochondria show no indication of damage. However after 2-3 days treatment with EGF the mitochondrial network changes its appearance, as mitochondria that normally have an elongated structure acquire a rounded shape with closely packed stacks (Fig. 5B, arrow, Fig. 8B, thick arrow). In parallel, the cells also change their morphology, becoming rounded with some membrane blebbing. In some cells, Bax is not only evenly distributed on the mitochondrial membrane, but also begins to form aggregates and to coalesce at mitochondrial fission sites (Fig. 5C, arrows), as has been described for staurosporine-induced apoptosis (Karbowski et al., 2002). Eventually, Bax completely aggregates in fission sites (Fig. 5D, arrow), which coincides with cell death, as determined by morphological criteria.

EGF-dependent Bax translocation to the mitochondria has been confirmed using another cell line, A431/ErbB2/Bax-mito, which is stably co-transfected with pDsRed2-Mito vector and Bax*GFP vector. Because Bax and mitochondria are labeled with green and red fluorescent proteins, respectively, no antibody staining was employed and cells were examined, after fixation, using confocal microscopy. Since these cells express exogenous Bax, its redistribution, and cell death, occur more quickly (usually in 24 hours) than in A431/ErbB2-mito cells. Analysis of Bax redistribution induced by EGF in these cells shows that Bax relocation does not occur simultaneously in the entire cell. Rather, Bax redistribution to the mitochondria first occurs in one part of a cell and then gradually appears throughout the cell (Fig. 5E). At the same time, Bax complexes accumulate along individual mitochondria such that the Bax pattern of redistribution reflects, in fine detail, the pattern of the mitochondrial network (Fig. 5F). At the final stage of apoptosis, Bax coalesces to the mitochondrial fission sites as was observed in A431/ErbB2-mito cells (Fig. 5G).
Bax translocation in cells treated with EGF results in a loss of MMP

Bax translocation and activation in cells usually leads to the formation of pores in the mitochondrial outer membrane and a loss of MMP. To determine whether EGF-induced Bax translocation coincides with a loss of MMP in this system, A431/ErbB2/Bax*GFP cells were incubated with EGF for 16-24 hours and then stained with MitoTracker CMTMRos dye, which is sequestered by actively functioning mitochondria. Under these conditions mitochondria of cells that still show Bax evenly distributed in the cytosol were brightly stained with the mitochondrial dye, similar to those in the control untreated cells (Fig. 6). However, if Bax translocation to foci occurs, the MMP is quickly lost, as judged by the loss of the mitochondrial staining. After 16-24 hours incubation with EGF, there is an almost 100% correlation between Bax translocation to foci and the loss of MMP. Interestingly, some cells with a partial translocation of Bax to foci have a partial loss of the MMP in that area of the cell, which contains translocated Bax (Fig. 6, arrow).

Bcl-xL prevents EGF-induced apoptosis

It is known that anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-xL, can block Bax- and Bak-induced apoptosis through sequestration of the BH3 domain-only protein (Cheng et al., 2001). We have found that SKBr3/EGFR and A431/ErbB2 cells contain low amounts of Bcl-2 and moderate levels of Bcl-xL (data not shown). During EGF-induced apoptosis, the levels of Bcl-xL in A431/ErbB2 cells and SKBr3/EGFR gradually decline, as detected by western blotting (Fig. 7A). Therefore, if Bax plays a significant role in cell death stimulated by EGF, the presence of anti-apoptotic Bcl-2 proteins should abolish or decrease the rate of apoptosis. A431/ErbB2 cells were transfected with a pIRES2-EGFP vector containing Bcl-xL, and several independent cell lines stably expressing this protein were selected. As shown in Fig. 7C, cellular responses to EGF are completely different in A431/ErbB2 and A431/ErbB2/Bcl-xL cells. A431/ErbB2 cells treated with EGF have a tendency to form dome-like structures consisting of cell clusters (Fig. 2A). In 2-3 days most cells on the top of these structures lose contact with the surface, or with other cells, and die. In contrast, although
A431/ErbB2/Bcl-xL cells evidently become activated in the presence of EGF, having many vacuoles and neurite-like processes of the plasma membrane, they remain spread on the extracellular matrix, continue to grow, and eventually form a monolayer (Fig. 7B).

**A431/ErbB2/Bcl-xL cells evidently become activated in the presence of EGF.**

Cytochrome c remains predominantly trapped inside the mitochondria during EGF-induced apoptosis

Bax translocation to the mitochondrial membrane leads to a pore formation and the release of cytochrome c and several other proteins from the mitochondrial intermembrane space. A western blot of the mitochondrial and cytosolic fractions of SKBr3/EGFR cells demonstrates that EGF induces the release of a small amount of cytochrome c from the mitochondria into the cytosol (Fig. 8A). Similar results have been obtained for A431/ErbB2 cells. However, there is no significant decline of cytochrome c in mitochondrial fractions.

To detect changes in the cytochrome c redistribution, A431/ErbB2-mito cells were treated with EGF and stained with a cytochrome c antibody. Cytochrome c is colocalized with mitochondria in the control cells and in most cells treated with EGF for 8-24 hours of (Fig. 8B, thin arrows). In cells with a distorted compressed mitochondrial network observed during the late stages of apoptosis, cytochrome c indeed can be detected in cytosol (Fig. 8B, thick arrow). However, as can be seen in Fig. 8B, and was consistently observed in other cells, a collapsed mitochondrial network of these cells still entraps a large amount of the cytochrome c inside.
responses to EGF stimulation in normal keratinocytes and we observe in A431 cells expressing Bax*GFP. The cellular actively proliferating keratinocytes are more vulnerable to ErbB-2 (Lewis et al., 2003a; Lewis et al., 2003b). Interestingly, manner that requires the kinase activity of both EGFR and keratinocytes significantly increases the rate of cell death in a ErbB-3. Activation of ErbB receptors by UV light in Normal skin keratinocytes co-express EGFR, ErbB-2 and cultures of epithelial cells induces cell death through apoptosis. This can be regarded as a protective mechanism receptor expression stimulation with EGF consistently induces apoptosis, which can be attributed to the expression of both receptors is low to moderate. At higher levels of expression should synergistically stimulate proliferative pathways increasing the chance of transformation. However, we have found that this is true only if the level of expression of both receptors is low to moderate. At higher levels of receptor expression stimulation with EGF consistently induces apoptosis, which can be regarded as a protective mechanism against cellular transformation.

Recent data suggest that activation of EGFR in HaCaT untransformed keratinocyte cell line and even in primary cultures of epithelial cells induces cell death through apoptosis. Normal skin keratinocytes co-express EGFR, ErbB-2 and ErbB-3. Activation of ErbB receptors by UV light in keratinocytes significantly increases the rate of cell death in a manner that requires the kinase activity of both EGFR and ErbB-2 (Lewis et al., 2003a; Lewis et al., 2003b). Interestingly, actively proliferating keratinocytes are more vulnerable to apoptosis than differentiated or senescent cells, similar to what we observe in A431 cells expressing Bax*GFP. The cellular responses to EGF stimulation in normal keratinocytes and several tumor cell lines are biphasic, which implies that high doses of EGF can inhibit cell growth in primary epithelial cells at a certain stage of their development. Co-expression of both EGFR and ErbB-2 in keratinocytes may be responsible for a decreased threshold for activation of apoptotic pathways.

Activation of the EGF receptor is also responsible for cell death in GH4C1 pituitary cells. Cell death induced by EGF is caspase-independent and involves a loss of MMP, but does not involve the release of cytochrome c or AIF from mitochondria. EGF-induced cell death in GH4C1 cells can be blocked by a Bcl-2 overexpression (Fombonne et al., 2004). The characterization of apoptosis in GH4C1 cells closely resembles the morphological and biochemical features of EGF-induced apoptosis in A431/ErbB-2 and SKBr3/EGFR cells. However, the data reported herein are the first to demonstrate the role of EGF-induced Bax activation and translocation in growth factor-induced apoptosis and may also apply to previously described systems in other cell types, including keratinocytes and GH4C1 cells.

Our results show that EGF induces relocation of Bax in cells that express elevated levels of EGFR and ErbB-2. Bax redistribution in response to EGF is a two-step process, which agrees with observation made using cells treated with staurosporine (Capano and Crompton, 2002). During the first step Bax changes its intracellular location, first concentrating in areas surrounding some mitochondria and then associating with nearly all mitochondria. Previous studies have shown that after induction of apoptosis by staurosporine Bax translocates to the mitochondrial outer membrane and almost instantly concentrates into punctate loci (Karbowski and Youle, 2003). In contrast, we have found that during EGF-induced apoptosis there is a gradual translocation of Bax to the mitochondrial outer membrane between 8-24 hours and its accumulation in foci. The presence of Bax on the mitochondrial membrane can be detected in most A431/ErbB2mito cells at 24-48 hours after addition of EGF. At that time most mitochondria still have their usual elongated shape and there is no indication of a loss of MMP. This gradual accumulation of Bax correlates with changes in the shape of mitochondria, which become rounded but still have MMP. Previously, it was been suggested that Bax translocation to foci coincides with its activation (Nechushtan et al., 2001). However, we have found that during early stages of apoptosis translocated Bax can be detected with 6A7 antibody, which indicates that Bax, present on the mitochondrial membrane, is already activated before its accumulation in foci.

Eventually these changes are followed by a second phase, which includes the more rapid formation of large Bax aggregates as described for staurosporine-treated cells (Wolter et al., 1997; Capano and Crompton, 2002) and correlates with a loss of MMP. This process usually extends throughout the cytosol from one discrete part of a cell to another. This suggests that the mitochondrial network comprises several separate sections of interconnected individual mitochondria, which are separated from each other. Since these events are significantly accelerated in A431/ErbB2/Bax cells, the rate of EGF-induced apoptosis correlates with Bax intracellular levels indicating that there is no saturation of docking sites for endogenous Bax on the mitochondrial membrane and that the intracellular level of Bax is a rate-limiting factor in EGF-induced apoptosis.

An interesting and intriguing feature of EGF-induced apoptosis...
apoptosis is its independence from caspase activation. Currently, as a result of observations made predominantly on staurosporine-treated cells, pore formation induced by Bax oligomerization in the mitochondrial outer membrane is regarded as a step of the apoptotic program that leads to the release of cytochrome c and other mitochondrial proteins into the cytosol with subsequent caspase activation (Arnoult et al., 2003; Riedl and Shi, 2004). However, our data show that during EGF-induced apoptosis the release of cytochrome c into the cytosol is limited and that most cytochrome c remains entrapped within mitochondria even after the loss of MMP. The low amounts of released cytochrome c correlate with a low level of caspase activation observed during EGR-induced apoptosis. Our data also indicate that although there is some activation of caspases 3 and 7 in cells undergoing apoptosis, the presence of the pan-caspase inhibitor z-VAD-FMK does not block cell death. Since caspase 1 has been implicated in EGF-induced apoptosis in A431 cells (Chin et al., 1997), we also employed a caspase 1 inhibitor and other inhibitors of individual caspases, including caspase 3-6, 8, 9, 13. None of these caspase inhibitors blocked EGF-induced apoptosis in A431/ErbB-2, SKBr3/EGFR or several other cell lines (data not shown). These findings imply that activation of caspases does not play an important role in EGF-induced apoptosis. Even so, EGF-induced cell death has obvious hallmarks of apoptosis, such as membrane blebbing, retraction of processes, nuclear fragmentation, chromatin condensation and decrease in the volume of cytosol, which suggests that a loss of MMP could be sufficient for induction of cell death with the morphological characteristics of apoptosis.

The opening of mitochondrial pores and release of AIF and endonuclease G, which induce nuclear apoptosis, could be responsible for DNA fragmentation and chromatin condensation. However, a recent publication suggests that during EGF-induced caspase-independent apoptosis in GH4C1 cells there is no activation of AIF (Fombonne et al., 2004). Another recent publication reported that caspase inhibition prevents the mitochondrial release of AIF and endonuclease G downstream of Bax-mediated opening of pores in the mitochondrial membrane (Arnoult et al., 2003). These data imply that there are other unknown mitochondria-related pathways activated during executionary phases of EGF-induced apoptosis, which are independent from caspase activation.

Transformation of most cell types by oncogenes, including EGFR, ErbB-2, Src and Ras, often sensitizes cells to stress-induced apoptosis and forms the basis of cytotoxic chemotherapy (Benhar et al., 2001). Previous studies showed that EGF-induced apoptosis is mediated by p38 MAPK (Tikhomirov and Carpenter, 2004). Both in vitro transformed cells and tumors have enhanced sensitivity to chemotherapeutic drugs, gamma radiation and other agents that activate p38 MAPK. A number of chemicals, such as cisplatin, aplidin, arsenic trioxide (Maeda et al., 2001), phytosphingosine (Park et al., 2003), ceramide (Carracedo et al., 2004) and aglaistatin (Yuan et al., 2003; Hausott et al., 2004) are known to increase p38 MAPK activity and selectively induce apoptosis in transformed cells. Activation of p38 MAPK by these drugs often induces Bax translocation to the mitochondria and is accompanied by the increased production of reactive oxygen species (ROS) that results in damage to the mitochondrial respiratory chain.

Cisplatin, a DNA-damaging agent, is widely used in chemotherapy. Recent studies have shown that cisplatin triggers the activation of p38 MAPK in tumor cells, which promotes Bax translocation to mitochondria and the loss of mitochondrial transmembrane potential, resulting in apoptosis (Yuan et al., 2003). Pretreatment with p38 inhibitors prevents cisplatin-induced apoptosis in transformed cells. Activation of p38 MAPK by cisplatin is markedly enhanced in NIH3T3 cells transfected with EGFR or ErbB-2. In these cells cisplatin stimulates activation of the EGFR tyrosine kinase. Apoptosis in these cells is blocked by specific inhibitors of p38 MAPK, antioxidants and inhibitors of the mitochondrial respiratory chain (Benhar et al., 2001).

Aplidin, an anti-tumor drug of marine origin, induces apoptosis in several cancer cell lines, including MDA-MB-231 breast cancer cells and renal cancer cell lines. In cell lines expressing the EGFR, aplidin activates EGFR, which in turn leads to the activation of p38 MAPK and JNK and a loss of MMP. Specific inhibitors of EGFR and p38 MAPK prevent apoptosis induced by the drug (Cuadrado et al., 2003).

The present data may explain some controversial results in experiments involving inhibition of ErbB receptor tyrosine kinases. Our results imply that many anti-neoplastic drugs activate the same apoptotic pathway as that engaged during EGF-induced apoptosis. This suggests that EGFR and ErbB-2 co-expression and levels in a cell as well as the presence of Bcl-2 family members may determine the cellular outcomes of EGF-dependent signaling and may have important clinical implications.

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