Ligand-induced, p38-dependent Apoptosis in Cells Expressing High Levels of Epidermal Growth Factor Receptor and ErbB-2*

Received for publication, October 23, 2003, and in revised form, December 30, 2003
Published, JBC Papers in Press, January 7, 2004, DOI 10.1074/jbc.M31655200

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Increased expression of the epidermal growth factor (EGF) receptor (EGFR) and ErbB-2 is implicated into the development and progression of breast cancer. Constant ligand-induced activation of EGFR and ErbB-2 receptor-tyrosine kinases is thought to be involved in the transformation of fibroblasts and mammary epithelial cells. Data herein show that ligand stimulation of cells that express both the EGFR and the ErbB-2 may result either in cell proliferation or apoptosis depending on the expression levels of EGFR and ErbB-2. Mammary tumor cells that express low levels of both receptors or high levels of ErbB-2 and low levels of EGFR survive and proliferate in the presence of EGF. In contrast, fibroblastic cells or mammary tumor cells, which co-express high levels of EGFR and ErbB-2 invariably undergo apoptosis in response to EGF. In these cells persistent activation of p38 MAPK is an essential element of the apoptotic mechanism. Also, the data implicate a p38-dependent change in mitochondrial membrane permeability as a downstream effector of apoptosis. Ligand-dependent apoptosis in cells co-expressing high levels of EGFR and ErbB-2 could be a natural mechanism that protects tissues from unrestricted proliferation in response to the sustained activation of receptor-tyrosine kinases.

EGFR/ErbB-1 and ErbB-2 are type I receptor-tyrosine kinases characterized by the presence of an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic region that includes a tyrosine kinase domain. The extracellular domain of the EGFR is recognized by seven distinct ligands that are related in primary sequence, which results in tyrosine kinase activation (1). In response to ligand stimulation, the EGFR forms homo- or heterodimers as part of the activation and signaling mechanism (2, 3). ErbB-2 has no known direct ligand but can be activated through heterodimerization with EGFR or other members of the ErbB family. Studies of the interactions between ErbB receptors have shown that ErbB-2 is the preferred partner for heterodimerization and thereby potentiates transformation and pro-survival signaling pathways (2–6).

Ligand binding induces the rapid internalization of the EGFR and its subsequent lysosomal degradation (7). In contrast, ErbB-2 transactivation by the activated EGFR does not stimulate rapid internalization of ErbB-2 (8–11). Removal of transactivated ErbB-2 from the cell surface proceeds slowly through a combination of intracellular degradation mechanisms, including lysosomes, the proteasome, and other intracellular proteases (10, 12–13). Heterodimerization of ErbB-2 and the EGFR actually impedes the rapid ligand-dependent internalization of the EGFR and, thereby, results in increased activation of signaling pathways (4, 9, 14).

Increased EGFR or ErbB-2 expression or structural alterations in either receptor are frequent in human malignancies (2, 15–17). Overexpression of the EGFR is observed in a significant proportion of brain tumors (18–20). Also, glioblastomas and other tumors often express a deletion mutation in the EGFR gene that constitutively activates the receptor (2). Usually EGFR overexpression results in cellular transformation only if the presence of a ligand, for example transforming growth factor α (21, 22), accompanies the increase in the receptor level. ErbB-2 overexpression as a result of gene amplification has been detected in breast, ovarian, prostate, and other cancers (2, 23, 24). ErbB-2 overexpression is often associated with a more aggressive form of disease and poorer prognosis (2, 15, 16, 25). Interestingly, more advanced invasive carcinomas have lower levels of ErbB-2 expression than early benign forms of breast cancer, which suggests that there is selection against ErbB-2 overexpression during the early stages of tumorigenesis (15, 26). Several clinical studies demonstrate that co-expression of EGFR and ErbB-2 in tissues is associated with a poorer prognosis than expression of only one of these molecules (27–29). The conclusion usually drawn is that sustained activation of EGFR and ErbB2 leads to the transformation of cells that have elevated levels of these receptors.

Although EGFR and ErbB-2 are usually associated with increased cell proliferation it has also been reported that either receptor may activate an apoptotic program (30). For example, EGF stimulates cell death in A431 or MDA-468 cells (31, 32). EGF stimulation, the EGFR forms homo- or heterodimers as part of the activation and signaling mechanism (2, 3). ErbB-2 has no known direct ligand but can be activated through heterodimerization with EGFR or other members of the ErbB family. Studies of the interactions between ErbB receptors have shown that...
Apoptosis in Cells Co-expressing EGFR and ErbB-2

During each pregnancy cycle in mammmary epithelial cells, there are mechanisms that define cellular responses initiated by ErbB ligands. Because expression of high levels of EGFR and ErbB-2 is implicated in cell transformation as well as in ligand-induced apoptosis, it is important to identify the parameters by which stimulation of EGFR and ErbB-2 results in cell proliferation or cell death. Although the signaling mechanisms that underlie the capacity of the EGFR and ErbB-2 to stimulate cell proliferation have been intensively investigated, the mechanisms that lead to the apoptotic response by these receptors are considerably less clear. Herein we present data showing that co-expression of elevated levels of EGFR and ErbB-2 in several cell lines invariably results in induction of ligand-dependent cell death, which in all cases is mediated by p38 MAPK.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibody to the ErbB-2 cytoplasmic domain (Ab3) was from Oncogene Science, polyclonal antibodies to phosphorylated p38 MAPK, MKK3/6, MAPKAPK2, and HSP27 were from Cell Signaling Technology, p38 MAPK antibody and EGFR antibody were from Santa Cruz Biotechnology. The EGFR fused to GFP (pEGF-C1 vector) was a generous gift of Dr. Alexander Sorkin (University of Colorado Health Science Center) (40), and pcDNA3.1+ and pcDNA3.1+ hygro vectors were obtained from Invitrogen. The EGFP vector containing pDsRed2-Mito vector was purchased from BD Biosciences and used as a template to obtain RFP DNA by PCR. pcRES2-EGFP vector was also obtained from BD Biosciences. Goat anti-mouse antibody cross-linked horseradish peroxidase was from Chemicon Laboratories Inc., whereas LipofectAMINE was purchased from Invitrogen. The p38 MAPK inhibitors SB203580, 202190, and SKF 86002, MEK1 inhibitors U0126, PD98059,Ralf inhibitor, phosphatidylinositol 3-kinase inhibitor, wortmannin, Akt inhibitor, p70S6K protein kinase inhibitor rapamycin, general serine/threonine kinase inhibitor H7, cyclin kinase inhibitor olomoucine, protein kinase C inhibitors rottlerin and bisindolylmaleimide, c-Jun NH2-terminal kinase inhibitors I and II, radicicol, benzoxyl-carbonyl-Val-fluoromethyl ketone, genistein, herbinycin, geldanamycin, EGFR tyrosine kinase inhibitor Compound 56, AG1478, and ErbB2 inhibitor AG825 were purchased from Calbiochem.

Cell Lines and Cell Culture—NIH3T3 cells stably expressing EGFR (CL17 and SAA cells, gift of Dr. L. Beguinot and Dr. Paolo Di Fiore, respectively) were transfected with ErbB-2/pIRES2-EGFP construct containing an internal ribosome entry site (IRES) sequence and separately expressing GFP as a marker. For transfection experiments cells were grown to ~80% confluence and transfected with LipofectAMINE according to the manufacturer’s recommendations. The cells were then grown in 80% confluent monolayers in the presence of G418. Several drug-resistant clones were selected. Clones with high levels of ErbB2, as confirmed by Western blot, were used in experiments. HEK293 cells were transfected with an ErbB-2/RFP construct, and hygromycin-resistant cell clones were selected. Then cells were transfected with construct containing EGFR tagged at the carboxy-terminal with GFP and selected in the presence of G418.

SKBr3 and BT474 cells were transfected with a construct containing EGFR-GFP. After transfection cells were grown in the presence of G418 for 2 weeks, and then cells were sorted using GFP as a marker. Five clones of SKBr3/EGFR cells and three clones of BT474/EGFR cells were established several independent cell lines from these populations. That this selection resulted in increased levels of ErbB-2 is shown in Fig. 1A.

The same strategy was employed to prepare the ErbB-2 expression vector tagged with RFP. The RFP sequence was amplified using a 5′ primer, TCG TGG GAT GGC CCG GTC GGC ACC ATG GCC TCC TCC GAG, and a 3′ primer, CTA GAG TCG TCT AGA CTA CAG GGA CAG GTG GTG GCG GCC CTC, containing Sall and XbaI restriction sites, respectively. The ErbB-2 sequence was inserted into the pAL-1Hygro vector through NheI and XbaI restriction sites, and the final ligation product was inserted into this vector through KpnI and XbaI restriction sites.

DNA Ladder and Terminal Deoxynucleotidyltransferase DNA Fragmentation Assay—SKBr3/EGFR cells were grown on 60-mm dishes or Titerter chamber slides and treated with EGF (100 ng/ml) for 2 days. A TdT-FragEL™ DNA ladder isolation kit (Oncogene) was used according to the manufacturer’s recommendations. For terminal dUTP nick-end labeling assay cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min, and DNA fragmentation was detected using the TdT-FragEL™ DNA fragmentation detection kit (Oncogene). All procedures were performed according to the manufacturer’s recommendations.

RESULTS

Co-expression of Exogenous EGFR and ErbB-2 in NIH3T3 and HEK293 Cells—To assess a possible role of EGFR and ErbB-2 co-expression in cellular responsiveness to EGF, we established several stable cell lines containing high levels of both receptors. NIH3T3 cells that stably express high levels of human EGFR (CL17 and SAA) were transfected with the pIRES2-EGFP vector containing the human ErbB-2-encoding sequence. Because this vector contains an IRES and allows expression of GFP as a marker separate from EGFR, we used flow cytometry to select cells with high levels of GFP and established several independent cell lines from these populations. That this selection resulted in increased levels of ErbB-2 is shown in Fig. 1C.

Co-expression of ErbB2 and EGFR results in increased ErbB-2 levels on EGF-dependent cell growth. EGF does not induce apoptosis in the parental CL17 cells (Fig. 1A). However, the addition of EGF to CL17 cells that express high levels of both EGFR and ErbB-2 (CL17/ErbB-2) results in a changed (more rounded) morphology, loss of contact with extracellular matrix, nuclear shrinkage, and eventually cell death in 4 days (Fig. 1A). Similar data were obtained with SAA cells and SAA/ErbB-2 transfectants.

This observation was confirmed using HEK293 cells that do not express high levels of endogenous ErbB receptors. Vectors containing either EGFR tagged with GFP attached to its carboxyl terminus (40) or ErbB-2 with GFP attached to its carboxyl terminus were used to doubly transfect HEK293 cells. Several independent cell lines containing high levels of both...
receptors, as determined by Western blot (Fig. 1C), were selected, and membrane localization of EGFR and ErbB-2 was verified by fluorescent microscopy. Incubation of these HEK293/EGFR/ErbB-2 cells with EGF induced cell death in 70–80% of cell population in 4 days (Fig. 1B). However, the growth of control cells was not significantly changed by the addition of EGF. These data indicate that enhanced levels of EGFR and ErbB-2 in two experimental cell systems do not necessarily result in the stimulation of cell proliferation after the addition of EGF. Rather the addition of EGF to these cells results in cell death.

**Co-expression of EGFR and ErbB-2 in Carcinoma Cells—** Several mammary carcinoma cell lines, such as SKBr3 and BT474, have high levels of ErbB-2 and low levels of EGFR (41), and stimulation with EGF does not result in cell death. To determine whether increased expression of EGFR would sensitize these cells to ligand-induced death, SKBr3 and BT474 cells were transfected with GFP-tagged EGFR, and stable cell lines were established. During selection most cells with high levels of EGFR died, but several cell lines that expressed elevated levels of EGFR were established (Fig. 2C). In contrast to the parental cells, which exhibited no detectable level of cell death under these conditions, about 70% of BT474/EGFR and almost 100% of SKBr3/EGFR cells died 4–5 days after the addition of EGF (Fig. 2, A and B).

We also stably transfected ErbB-2 into A431 cells, which express high levels of EGFR and low levels of ErbB-2 (42). The increased level of ErbB-2 expression (~5-fold) was demonstrated by Western blot (data not shown). Although it is known that EGF stimulates apoptosis in A431 cells (31), we found that ~10–20% of cells usually survived treatment with EGF for several days and formed clones viable in the presence of EGF. In contrast, all A431 cells stably transfected with ErbB-2 cells die upon treatment with EGF, and no viable clones could be detected (data not shown).
The cells were treated with EGF (100 ng/ml) for 4 days. ErbB-2 chimeric receptor were grown in DMEM containing 1% FBS. NIH3T3 cells expressing high levels of EGFR/ this inhibitor at all concentrations tested (20

Sustained Activation of the ErbB-2 Kinase Results in Cell Death—The above results suggest that ligand-dependent transactivation of ErbB-2 could be a mechanism by which EGF-dependent cell death is provoked. To test this possibility we used an NIH3T3 cell line that expresses a high level of a chimeric EGFR (ectodomain plus transmembrane domain)/ ErbB-2 (cytoplasmic domain) receptor. These cells express ~7 x 10^6 chimeric receptors per cell, and the ErbB-2 tyrosine kinase is activated by EGF (43). The cells were incubated in 1% FBS and treated with EGF for 4 days. The data in Fig. 3 show that under these conditions the addition of EGF resulted in high level of cell death (80–90%). This result indicates that sustained activation of the ErbB-2 kinase is sufficient to mediate cell death.

EGF Induces Apoptosis in Cells That Overexpress EGFR and ErbB-2—To further characterize the cell death response induced by the addition of EGF, hallmarks of apoptosis were examined. The data in Fig. 4 show that SKBr3/EGFR cells treated with EGF displayed apoptotic morphology characterized by DNA fragmentation as detected by ladder formation (Fig. 4A), terminal dUTP nick-end labeling assay (Fig. 4B), and nuclear fragmentation visualized by Hoechst staining (Fig. 4C). These cells also exhibited reduced cytoplasmic space, chromatin condensation, and detachment from the extracellular matrix. Because it has been suggested that apoptosis in A431 cells treated with EGF depends on caspase activation (44), the pan-caspase inhibitor benzoylcarbonyl-Val-Asp-fluoromethyl ketone was employed to prevent ligand-induced cell death in A431, A431/ErbB-2 cells, SKBr3/EGFR cells, or NIH3T3 cells that express the EGFR/ErbB-2 chimeric receptor. In each case this inhibitor at all concentrations tested (20–100 μM) did not abrogate EGF-induced apoptosis. Also, there was no significant activation of caspase 3 (detectable by either Western blot with antibody against activated caspase 3 or formation of cleaved fluorescent substrate detected using the caspase 3 intracellular activity assay Kit, Calbiochem) in cells undergoing EGF-induced apoptosis.

The p38 MAPK Inhibitors Block EGF-induced Apoptosis—In attempt to determine the mechanism of EGF-induced apoptosis ~50 pharmacologic inhibitors of specific signaling pathways were tested. EGF-induced cell death in SKBr3/EGFR cells was not blocked by inhibitors of the extracellular signal-regulated kinase 1/2 pathway (U0126, PD98059, Raf1 inhibitor), the phosphatidylinositol 3-kinase/Akt pathway (wortmannin, Akt inhibitor), general serine/threonine kinases H7, cyclin-depend-ent kinases (olomoucine), protein kinase C (rotterlin, bisindolylmaleimide), p53 (pifithrin), p70S6 kinase (rapamycin), metalloproteases (GM1489, MMP II, BB3103, BB89), serine proteases (4-(2-aminoethyl)benzenesulfonyl fluoride, aprotnin), calpain (E64d), trypsin-like proteases and chymotrypsin (aprotinin, 1-chloro-3-tosylamido-7-amino-2-heptanone, 1-1-to-sylamido-2-phenylethyl chloromethyl ketone), tyrosine kinase (herbimycin, geldanamycin), or Jun kinase (c-Jun NH2-termin-al kinase inhibitors I and II, Calbiochem). Among tyrosine kinase inhibitors only genistein (100 μM) and the specific EGFR/ErbB-2 kinase inhibitor PKI 116 (200 nM) efficiently blocked apoptosis.

However, the p38 MAPK-specific inhibitors SB203580 or SB202190 (10 μM) completely prevented cell death induced by EGF in cell lines that co-express EGFR and ErbB-2 (Fig. 4 and data not shown). In addition, these p38 MAPK inhibitors completely blocked apoptosis induced by EGF in A431 cells. The capacity of EGF to inhibit proliferation of SKBr3/EGFR cells and the influence of the p38 MAPK inhibitor SB203580 is shown quantitatively in Fig. 5. In the absence of added EGF the cells proliferated over the period of 8 days. In the presence of EGF, however, proliferation was suppressed, and the remaining cells were, in fact, dead cells. The addition of EGF and SB203580 prevented the cell death induced by EGF and restored cell proliferation to near control levels. EGF-induced autophosphorylation of the EGFR or ErbB-2 or activation of extracellular signal-regulated kinase 1/2, detected with phos- pho-specific antibodies, in SKBr3 cells were not significantly decreased in the presence of SB203580, which suggests specific inhibition of the p38 MAPK pathway (data not shown).

These data show that EGF-induced apoptosis in cells expressing high levels of EGFR and ErbB-2 is mediated by activation of the p38 stress kinase and that co-expression of EGFR and ErbB-2 may significantly alter the EGF-dependent activation of p38 MAPK. When this possibility was tested EGF provoked a sustained activation of p38 MAPK in SKBr3/EGFR cells as shown in Fig. 6. Activation of p38 MAPK was detected at 10 min after the addition of EGF (Fig. 6A) and was sustained for 72 h (Fig. 6B). In contrast, no sustained activation of p38 MAPK could be detected in parental SKBr3 treated with EGF (Fig. 6C), although p38 MAPK levels were equivalent to that in SKBr3/EGFR cells. Therefore, in the presence of high levels of both EGFR and Erb-2, EGF induces activation of a signaling pathway that is not induced without co-expression of both receptors.

Because p38 MAPK is activated by MKK3 or MKK6 protein kinases (45, 46), the activation of these kinases was tested in SKBr3/EGFR cells treated with EGF. In accordance with the activation of p38 MAPK by EGF in these cells, the results shown in Fig. 7A demonstrate a prolonged activation of MKK3/6 in EGF-treated SKBr3/EGFR cells but not in parental SKBR3 cells (Fig. 7B). Also, downstream targets of p38 MAPK were tested for EGF-dependent activation in SKBr3/EGFR cells. Activated p38 MAPK phosphorylates and activates its endogenous substrate, MAPKAPK2 protein kinase (47), in SKBr3/EGFR cells, as shown in Fig. 7C. MAPKAPK2 in turn is known to phosphorylate HSP27, a small heat shock protein that participates in the actin remodeling (48, 49). This is demonstrat-ed for EGF treatment of SKBr3/EGFR cells (Fig. 7D). The addition of SB203580 (10 μM) to SKBr3/EGFR cells prevent EGF-induced phosphorylation of MAPKAPK2 (Fig. 7C) and HSP27 (Fig. 7D), which confirms the inhibition of p38 MAPK by SB203580 in these cells.

Effect of EGF on Expression of Cell Cycle Inhibitors—It has been reported that EGF stimulation of A431 cells increases intracellular levels of the cyclin-dependent kinase inhibitor p21 (50). Western blot analysis was employed to examine the status of p21 in SKBr3/EGFR cells undergoing ligand-dependent apoptosis. As shown in Fig. 8A, the addition of EGF resulted in the up-regulation of p21 levels with maximal levels achieved after 24 h. Also, these data show that the addition of the p38 MAPK inhibitor SB203580 prevented EGF-induced p21 up-regulation, indicating that p21 accumulation is dependent on p38 MAPK activation. Also, stimulation with EGF increased intracellular
levels of the cell cycle inhibitor p27 in a p38-dependent manner (Fig. 8B). Two groups have reported that p53 is involved in apoptosis associated with increased ErbB-2 levels in MCF7 and IGROV1 cells (34, 36). However, we detected a dramatic decline in p53 levels in SKBr3/EGFR and A431 cells after 24 h of incubation with EGF, which was partially prevented by SB203580 (Fig. 8C and data not shown). This result suggests that EGF-induced apoptosis in these cells does not depend on p53 accumulation.

**EGF Induces Loss of Mitochondrial Potential**—Induction of apoptosis often changes mitochondrial membrane permeability (51, 52). A multiprotein complex including porin, adenine nucleotide translocator, cyclophilin D, and other proteins is localized in both the inner and outer mitochondrial membranes and is known as a permeability transition pore (PTP). In response to an apoptotic signal, PTP opens channels in the mitochondrial membrane, leading to the loss of potential $\Delta \psi_m$ across the mitochondrial membrane. It has been reported that in B lymphocytes activated p38 MAPK is translocated to mitochondria, where it induces cytochrome c release into the cytoplasm (53).

We analyzed whether EGF-induced apoptosis in SKBr3 and A431 cells is related to the loss of mitochondrial potential. As shown by the data in Fig. 9, PTP opening was detected in SKBr3/EGFR and A431 cells treated with EGF but not in cells treated with EGF and SB203580. This assay uses MitoCapture cationic dye, which fluoresces differently in healthy and apoptotic cells. In healthy cells aggregates of MitoCapture are accumulated in mitochondria, emitting a red fluorescence. After mitochondrial PTP opening, monomers of dye remain in the cytoplasm and emit green fluorescence. Because EGFR is fused to GFP in SKBr3/EGFR cells this green cytoplasmic staining can be detected only in A431 cells. As shown in Fig. 9, the addition of EGF to A431 cells for 2 days resulted in a loss of the red punctate staining and an increased green cytoplasmic staining. Therefore, during ligand-induced apoptosis in SKBr3/EGFR and A431 cells there is a loss of $\Delta \psi_m$ and release of mitochondrial proteins into the cytoplasm.

**DISCUSSION**

We explored the biologic outcomes of growth factor signaling through EGFR/ErbB-2 heterodimers in cells of different origins that express elevated levels of both receptors. To explore this issue in a systematic fashion, several cell lines were established based on parental NIH3T3 or HEK293 cells. The paren-
tal 3T3 cells expressing EGFR (CL17 and SAA cells) proliferate in the presence of EGF. However, when CL17 and SAA cells are stably transfected with ErbB-2, the cells undergo apoptosis in response to EGF. Similar outcomes were obtained with HEK293 cells that after transfection express high levels of both EGFR and ErbB-2 as well as carcinoma lines, which endogenously express ErbB-2 (SKBr3 and BT474) or ErbB-1 (A431) and are subsequently transfected to express high levels of EGFR or ErbB-2, respectively. Equivalent results have been obtained with other agonists of the EGFR, for example transforming growth factor-β or betacellulin (data not shown).

This result may be explained by the impact of ErbB-2 on EGFR trafficking. After ligand binding EGFR is quickly removed from the cell surface, sorted to endosomes, and then to lysosomes (7). EGFR down-regulation quickly uncouples this receptor from activation of downstream signaling molecules. ErbB-2 has an alternative intracellular routing in the presence of EGF, undergoing slow endocytosis and remaining much longer on plasma membrane (8). In fact, the presence of ErbB-2 decreases the rate of EGFR down-regulation such that EGFR/ErbB-2 heterodimers remain longer on the cell surface compared with EGFR/EGFR dimers (9, 11, 14). This results in exaggerated signaling after the addition of EGF (4, 54, 55). The cellular outcome of sustained EGFR signaling through EGFR and ErbB-2 depends on the expression levels of both receptors. Our data show that cells expressing elevated levels of EGFR and ErbB-2 invariably die in response to EGF stimulation.

**FIG. 6.** EGF-induced activation of p38 MAPK pathway in SKBr3/EGFR cells (A and B) or control SKBr3 cells (C). The cells were incubated in the presence of EGF (100 ng/ml), EGF and SB203580 (10 μM), or vehicle for the indicated times. After incubation the cells were lysed, and aliquots containing equal amounts of protein were subjected to 10% SDS-PAGE and Western blotting (W.B.) with antibodies to phosphorylated (anti-ph) p38 MAPK. The presence of equal amounts of protein in each sample was confirmed with antibody against total p38 MAPK.

**FIG. 7.** EGF-induced activation of p38 MAPK pathway in SKBr3/EGFR cells. The cells were incubated in the presence of EGF (100 ng/ml), EGF and SB203580 (10 μM), or vehicle for the indicated times. After incubation the cells were lysed, and aliquots containing equal amounts of protein were subjected to 10% SDS-PAGE and Western blotting (W.B.) with antibodies to phosphorylated (anti-ph) p38 MAPK, MKK3/6, MAPKAPK2, HSP27. The presence of equal amounts of protein in each sample was confirmed with antibody against total p38 MAPK. A, C, and D show activation of the indicated protein kinases in SKBr3/EGFR cells. B shows the absence of MKK3/6 activation in control SKBr3 cells.

**FIG. 8.** Effect of EGF on levels of cell cycle inhibitors. SKBr3/EGFR cells were incubated in the presence of EGF (100 ng/ml), EGF and SB203580 (10 μM), or Me2SO for the indicated times. Afterward the cells were lysed, and aliquots containing equal amounts of protein were subjected to 10% SDS-PAGE and Western blotting (W.B.) with antibodies to p21 (A), p27 (B), or p53 (C). Immunoreactive bands were detected by ECL.
Ligand-induced death through activation of EGFR and ErbB-2 is not restricted to certain cell types and most likely activates the same apoptotic pathways in cells of different origins. Whether the EGF-dependent induction of apoptosis is mechanistically related to ErbB-2-provoked decrease of EGFR endocytosis and down-regulation is not at present known for the cell systems described herein.

Normal epithelial cells as well as cancer cells usually express more than one ErbB receptor (15, 56). During proliferative phases in the cyclic development of some organs, e.g. during mammary gland morphogenesis, there is a considerable increase in certain ErbB receptor levels in epithelial cells (57–59) that does not result in cellular transformation. Therefore, there are mechanisms that control proliferation of mammary cells driven by ErbB receptors and ligands and prevent transformation. It is possible that EGF or other ligands that are capable of inducing apoptosis stimulate proliferation of cells that express low levels of EGFR and ErbB-2 but induce apoptosis in cells expressing elevated levels of these receptors. Interestingly, most tumor cell lines express high levels of either EGFR or ErbB-2 but not both (41).

Although signaling through ErbB-2 is usually considered to provoke a proliferative response, several reports suggest that ErbB-2 transfection results in increased cell death if the cells

**Fig. 9.** PTP opening and loss of mitochondrial potential in the A431 and SKBr3/EGFR cells. A431 (A) or SKBr3/EGFR (B) cells were grown on Lab-Tek® 4-well chamber slides in the presence of EGF (100 ng/ml), EGF and SB203580 (10 μM), or vehicle for 3–4 days. Then the cells were incubated for 30 min in incubation buffer from a commercial kit containing MitoCapture dye diluted 1:2000. In the cells that lost the mitochondrial potential, red punctate mitochondrial staining (right panels) is replaced with green cytoplasmic staining (middle panels), which was visualized by fluorescent microscopy. The arrows indicate apoptotic cells that have lost mitochondrial potential.
shown that activated p38 MAPK directly binds and phospho-

promoter of p21waf1/cip1 and up-regulates this cyclin-dependent

STAT1, which recognizes specific responsive elements in the
cell) (31, 32). This effect has been attributed to the activation of

in SKBr3/EGFR cells. The level of p21 was maximal after

served p21 up-regulation in response to EGF in A431 as well as

mutated at codon 273 in A431 cells and is inactive, indicating

mitochondrial membrane permeability. In memory B lymphocyte-acti-

ErbB-2. Rather, sustained stimulation of the p38 MAPK path-

ligand-induced apoptosis in cells with high levels of EGFR and

pan-caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl

ptosis in these cells is characterized by a reduction in mito-

cell system.

express both EGFR and ErbB-2 undergo ligand-dependent apo-

sion of ErbB-2 in cells with mutated p53 or with a dominant

negative construct of p53 abrogated ErbB-2-dependent apopto-

is abrogated by the addition of glucocorticoids (33). Also, two

groups have found that stable transfection of ErbB-2 in the

constitutively active ErbB-2 mutant neu-T in the human mam-

and adenine nucleotide translocator and modulate gating prop-

erties of a protein complex regulating the PTP opening. How-

ever, whether phosphorylation of Bel-2 may be responsible for

mitochondrial membrane leakage is currently a controversial

issue. Other pro-apoptotic members of Bel-2 family may also be

involved in p38 MAPK-mediated apoptosis.

In summary, our data indicate that cells expressing elevated

amounts of EGFR and ErbB-2 proliferate in response to ligand

stimulation only if signaling through EGFR and ErbB-2 does not

exceed a certain threshold. However, strong sustained ac-

tivation of EGFR and ErbB-2 above this threshold activates

apoptotic pathways and leads to cell death.

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