Epidermal Growth Factor Receptor-dependent Control of Keratinocyte Survival and Bcl-x$_L$ Expression through a MEK-dependent Pathway*

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Previous work has shown that the epidermal growth factor receptor (EGFR) tyrosine kinase moiety provides protection to normal human keratinocytes against apoptosis. This protection is, at least in part, due to EGFR-dependent expression of the antiapoptotic Bcl-2 family member, Bcl-x$_L$. Here we focused on intracellular signaling pathways relevant to keratinocyte survival and/or Bcl-x$_L$ expression. By using pharmacological inhibitors and dominant negative expression constructs, we observed that phosphatidylinositol 3-kinase/AKT and phospholipase Cγ/protein kinase Cα activation were required for keratinocyte survival independently of EGFR activation or Bcl-x$_L$ expression. By contrast, MEK activity required EGFR activation and, as shown by use of the MEK inhibitor PD98059 and a dominant negative MEK construct, was necessary for Bcl-x$_L$ expression and survival. Consistent with an earlier study, blocking SRC kinase activities similarly led to down-regulation of Bcl-x$_L$ protein expression and impaired keratinocyte survival. In conclusion, our results demonstrate that EGFR-dependent MEK activity contributes to both Bcl-x$_L$ expression and survival of normal keratinocytes. Other signaling pathways (*i.e.* phosphatidylinositol 3-kinase/AKT and phospholipase Cγ/protein kinase Cα) are obligatory to keratinocyte survival but not to Bcl-x$_L$ expression, and control of these pathways by EGFR activation is not rate-limiting to normal keratinocyte survival.

Extracellular signals are essential to the survival of normal cells in multicellular organisms. Appropriate signals are transmitted in cell type-specific fashion by growth factor/cytokine, cell-matrix adhesion, or cell-cell adhesion receptors. Removal of essential survival signals triggers physiological cell death by default. Recent studies have implicated many cell surface receptors previously known to affect cell cycle progression and proliferation also in cellular survival. For example, normal human keratinocytes require activation of the epidermal growth factor receptor (EGFR)[1] by exogenous or endogenous

signals to survive (1–4). Specifically, EGFR blockade using a ligand-antagonistic monoclonal antibody results in enhanced susceptibility to cell death provoked by cellular stressors such as passaging (1) or UVB irradiation.[2] Several lines of evidence highlight a prominent role of Bcl-x$_L$, an anti-apoptotic Bcl-2 family member, in EGFR-dependent keratinocyte survival. First, activation of the EGFR on keratinocytes by exogenous or endogenous ligands is associated with up-regulation of Bcl-x$_L$ expression (2). Second, induction of cell death by EGFR blockade is a slow process requiring at least several days of EGFR blockade and is preceded by down-regulation of Bcl-x$_L$ (1). Third, forced expression of Bcl-x$_L$ in immortalized keratinocytes rescues these cells from cell death associated with EGFR blockade (4). EGFR-dependent tyrosine phosphorylation appears to be required for protection against cell death because EGFR-selective tyrosine kinase inhibitors of the tyrphostin class cause Bcl-x$_L$ down-regulation and cell death in a manner similar to the EGFR-antagonistic monoclonal antibody (2). Taken together, these earlier results implicate signal transduction pathways activated by EGFR-dependent phosphorylation events in keratinocyte survival.

The multiplicity of cell surface receptors activated by exogenous signals is contrasted by the relative uniformity of intracellular signaling pathways triggered by these receptors. For example, EGFR activation leads to activation of parallel signal transduction pathways including SRC, RAS, phosphatidylinositol (PI)-3 kinase, signal transducer and activator of transcription (STAT) 3, and phospholipase (PLC) γ in different cell types (reviewed in Ref. 5). The same signaling components are also affected by engagement of various other growth factor receptors and of adhesion receptors of the integrin family. Thus, integration of multiple signals from diverse growth factor and adhesion receptors must take place at a membrane-proximal location to generate parallel second messengers. By implication, signal strength for individual signaling pathways may be provided either by activation of multiple receptors by diverse ligands or by strong signaling by a single class of receptors. The former scenario is likely to apply to normal cells, whereas the latter may operate in malignant cells, which have become independent of certain extracellular signals for survival. In view of this interpretation, it is important to define the

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PI 3-kinase, phosphatidylinositol

3-kinase; mAb, monoclonal antibody; PKC, protein kinase C; PLC, phospholipase C; FACS, fluorescence-activated cell sorter; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; HA, hemagglutinin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TBS, Tris-buffered saline; TUNEL, terminal dUTP nick-end labeling; STAT, signal transducer and activator of transcription; IGR, insulin-like growth factor.

[2] M. Jost, F. P. Gasparro, and U. Rodeck, unpublished observations.

relative contribution of each single class of cell surface receptors to signal transduction events required for cellular survival.

In this study, we attempted to distinguish signaling pathways obligatory to keratinocyte survival, which depend on EGFR activation from those that are required independently of EGFR activation. By using a combination of small molecular weight inhibitors and dominant negative expression constructs, we probed the contribution of several signaling cascades including the SRC, PI 3-kinase/AKT, PLCγ/PKC, STAT, and MEK/MAPK pathways to keratinocyte survival and Bcl-x$_L$ expression. The effects of abrogating the function of specific signal transducers were related to the effects of EGFR blockade on keratinocyte survival and to Bcl-x$_L$ expression. We describe that PI3-kinase/AKT, PLCγ, and PKCα activities were essential to keratinocyte survival. However, these pathways did not depend on the EGFR tyrosine kinase activity to support keratinocyte survival and did not affect Bcl-x$_L$ expression. By contrast, MEK activity was dependent on EGFR activation and was required for sustained Bcl-x$_L$ expression and keratinocyte survival.

EXPERIMENTAL PROCEDURES

Materials and Cells—Properties of the EGFR antagonistic mAb 425 have been described earlier (6–9). The MEK inhibitor PD98059, PI 3-kinase inhibitor LY294002, and tyrphostins AG1478 and AG1295 were purchased from Calbiochem-Novabiochem. SRC inhibitors PP1 and PP2, the control compound PP3, PLCγ inhibitor U73122, and the control compound U73343 were purchased from Calbiochem or Biomol (Plymouth Meeting, PA). PKCα/β inhibitor Gö6976 and PKCδ inhibitor Rottlerin were purchased from Biomol. Antibodies to Bcl-x$_L$ were purchased from Transduction Laboratories (Lexington, KY), to β-actin from Amersham Pharmacia Biotech, and to α-tubulin from Oncogene Research Products (Boston, MA). Antibodies to signal transduction components (STAT3, phospho-STAT3, p42/44-MAPK, phospho-MAPK, AKT, phospho-AKT, phospho-PKCα/β, PKCα, and PLCγ) were purchased from Cell Signaling Technology (Beverly, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). HA tag antibodies were from Covance (Richmond, CA).

Normal foreskin keratinocytes and HaCaT cells were maintained in culture as described earlier (10). For treatment with inhibitors cells were seeded at subconfluency in complete MCDB medium (4). After attachment medium was replaced with base MCDB medium (4), supplemented with insulin as the only proteinaceous growth factor. Inhibitors were diluted from Me$_2$SO stocks directly into the culture medium. The Me$_2$SO concentration was adjusted to 0.5% in all conditions including controls. After 48 h cells were harvested for analysis as described below.

cDNA Constructs and Transfections—A plasmid containing dominant negative MEK1 cDNA (pMCL-MKK1-8E (11)) was kindly provided by Dr. Natalie Ahn. The MKK1-8E mutant carries a methionine substitution in the active center of the kinase domain (Lys-79) and is HA-tagged at the N terminus. MKK1-8E was cloned into pCEPTetP to create pCEPTetP-MKK1-8E. Constructs encoding dominant negative STAT3 mutants pCAGGS-STAT3D and pCAGGS-STAT3F were a generous gift from Dr. T. Hirano (12). In STAT3F, the C-terminal tyrosine (Tyr-705) has been changed to phenylalanine, which prevents phosphorylation of this mutant. The replacement of two glutamic acids (Glu-34 and Glu-35) in the DNA binding domain of the STAT3D mutant renders this protein unable to bind DNA. The Stat3D and Stat3F fragments are HA-tagged and were cloned as *NheI/Bam*HI fragments into pCEPTetP to create pCEPTetP-Stat3D and pCEPTetP-Stat3F. A dominant negative AKT-encoding plasmid was obtained from Dr. Thomas F. Franke (pCMV-HA-AKT(K179M)) (13). By replacing a lysine (Lys-179) with methionine in the kinase domain, it has been rendered inactive. HA-AKT(K179M) was subcloned into pCEPTetP.

Transfections were performed using Fugene 6 (Roche Molecular Biochemicals). Briefly, cells were seeded at a density of ~3 × 10³/cm² in complete MCDB medium and allowed to attach overnight. Transfections were performed 1 day after seeding using 0.5 µg of DNA and 1.7–2.0 µl of Fugene 6 per 10⁵ cells following the manufacturer's protocol. Selection was started 48–72 h after transfection using 0.2 mg/ml G419 (Mediatech, Herndon, VA), 0.1 mg/ml hygromycin B (Roche Molecular Biochemicals), and 2 µg/ml tetracycline (Sigma).

Reporter Gene Assays—Transient transfections using a 1.1-kilobase

pair Bcl-x$_L$ promoter fragment cloned into pGL3 (Promega) driving expression of luciferase were performed as described (14). Luciferase and β-galactosidase assays were performed using reagents from PharMingen (San Diego, CA) and Tropix Inc. (Bedford, MA) as described earlier (14).

Immunoblot Analyses—For Western blot analyses, attached cells were washed once in cold PBS and then lysed in 1× nonreducing Laemmli buffer followed by boiling for 3–5 min. Protein content was determined using the BCA method (Pierce). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Millipore). Membranes were treated with blocking buffer (5% dry milk in PBS or 5% dry milk, 0.05% Tween 20 (Sigma) in TBS). Primary antibodies were diluted in PBS or in TBS containing 5% BSA, 0.05% Tween 20 and incubated with membranes for 12 h at 4 °C followed by incubation (1 h at room temperature) in dilutions of horseradish peroxidase-conjugated secondary antibodies in the same buffers. Following antibody incubations membranes were washed in 0.5% Tween 20 in PBS or TBS. Signals were visualized by chemiluminescence using reagents from Pierce according to the manufacturer's instructions. After detection, blots were washed and inactivated by staining with SG substrate (Vector Labs) and used for further antibody incubations.

Kinase Assays—AKT and MAPK assays were performed using nonradioactive kits manufactured by New England Biolabs. Briefly, cells were treated for 48 h and lysed in 1× lysis buffer. Equal amounts of proteins were immunoprecipitated with either an immobilized phospho-MAPK antibody that only cross-reacts with the phosphorylated, *i.e.* active, form of MAPK or with an immobilized AKT antibody that recognizes both active and inactive AKT. The immobilized precipitated enzymes were then used for kinase assays using either ELK-1 (for MAPK) or GSK-3β (for AKT) followed by Western blot analysis with antibodies that allow detection and quantitation of phosphorylated substrates.

Apoptosis Detection Assays—To detect DNA nicks and breaks as markers of apoptotic cell death, TUNEL assays were performed. Briefly, floating and attached cells were collected and fixed in 70% ethanol at 4 °C. Prior to staining with Biotin-16-dUTP (Roche Molecular Biochemicals), cells were washed in PBS containing 1% BSA. Staining was performed using TdT enzyme and buffers purchased from Roche Molecular Biochemicals for 1 h at 37 °C. After the TUNEL reaction cells were stained with 2.5 µg/ml fluorescein isothiocyanate/ Avidin (Vector Laboratories, Burlingame, CA) in 4× SSC (1× SSC is 0.15 M NaCl, 0.015 sodium citrate), 0.1% Triton X-100, 5% (w/v) nonfat dry milk followed by a wash with PBS containing 1% BSA and 0.1% Triton X-100. Cells were analyzed without delay using a FACScan cytometer (Coulter, Fullerton, CA).

RESULTS

Effects of EGFR Blockade on Major Signal Transduction Pathways in Human Keratinocytes—To identify signal transduction pathways relevant to EGFR-dependent Bcl-x$_L$ expression or survival of human keratinocytes, we first determined the effects of EGFR blockade by EGFR-antagonistic mAb 425 on several pathways previously shown to be affected by EGFR engagement in different cell types. These included PI 3-kinase/ AKT (15, 16), MEK/MAPK (17, 18), STAT3 (19, 20), and PLCγ (21). These experiments were performed using immortalized keratinocytes (HaCaT) in a serum-free medium containing insulin as the sole exogenous growth factor to account for EGFR-dependent signaling events induced by autocrine EGFR ligands, several of which are known to be produced by cultured keratinocytes (22–25). In addition, these experiments were performed using steady-state conditions by maintaining cells for 24–48 h under the same media conditions. In select experiments EGF was added short term (5–10 min) to the medium to provide a strong receptor activation signal. As shown in Fig. 1*A*, EGFR blockade by mAb 425 used at 66 nM markedly reduced steady-state phosphorylation of the MAPK substrate ELK-1 in normal human keratinocytes. By contrast, EGFR blockade using mAb 425 had little effect on GSK-3 β phosphorylation (Fig. 1*B*); GSK-3 β is a major target of the serine/threonine kinase AKT (26). As controls we used the MEK-1 inhibitor PD98059 to inhibit ELK-1 phosphorylation
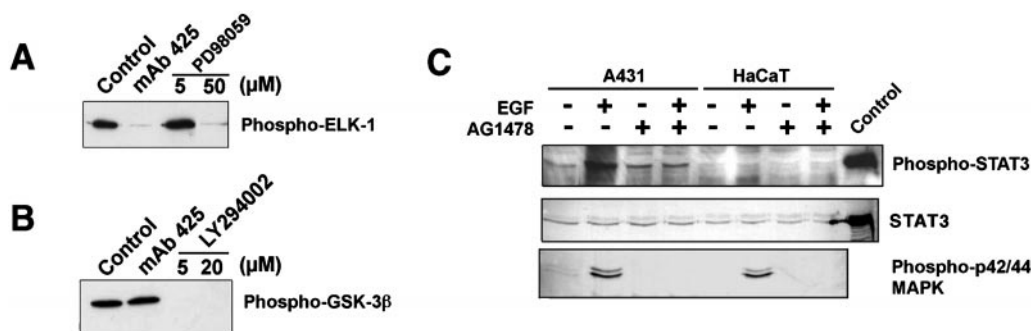
FIG. 1. **Effects of EGFR blockade on ELK-1, GSK-3β, and STAT3 phosphorylation in immortalized HaCaT keratinocytes.** *A, in vitro* phosphorylation of ELK-1 after treatment with mAb 425 (10 μg/ml) for 48 h; as a positive control the effects of the MEK inhibitor PD98059 at different concentrations are shown. *B, in vitro* phosphorylation of GSK-3β after treatment with mAb 425 for 48 h; as a positive control the effects of the PI 3-kinase inhibitor LY294002 at different concentrations are shown. *C,* expression and phosphorylation levels of STAT3 in HaCaT keratinocytes induced by short term EGF treatment (10 ng/ml; 5 min) in the presence and absence of EGFR inhibitor AG1478 (10 μM); STAT3 phosphorylation patterns of A431 cells exposed to the identical culture conditions are also shown. Control cells were HaCaT keratinocytes overexpressing STAT3D. The *bottom panel* shows EGF-dependent phosphorylation of p42/44 MAPK in HaCaT and A431 cells as determined by use of an antibody to phosphorylated MAPK.

and the PI 3-kinase inhibitor LY294002 to inhibit GSK-3β phosphorylation.

STAT3 was expressed prominently in HaCaT keratinocytes but was not phosphorylated under steady-state conditions (not shown). Similarly, short term stimulation of HaCaT cells (Fig. 1*C*) or normal primary keratinocytes (not shown) with EGF (10 ng/ml) failed to detectably induce STAT3 phosphorylation although it was associated with robust induction of MAPK phosphorylation that was inhibitable by the EGFR-selective tyrphostin AG1478. This was in contrast to A431 squamous carcinoma cells that contained comparatively high levels of constitutively phosphorylated STAT3 and in which STAT3 phosphorylation was further stimulated by exogenous EGF. Inhibition of the EGFR tyrosine kinase moiety by use of AG1478 abrogated the effect of EGF on STAT3 and MAPK phosphorylation in A431 cells but did not affect base-line STAT3 phosphorylation in these cells. Previously, STAT3 phosphorylation was observed in primary mouse keratinocytes after placing them into forced suspension culture (27). Consistent with these earlier findings we also observed STAT3 phosphorylation in primary keratinocytes placed in suspension culture (not shown). This result demonstrates that STAT3 phosphorylation was inducible in human keratinocytes albeit not through activation of the EGFR.

Similar to STAT3, we did not observe PLCγ phosphorylation in normal and immortalized keratinocytes in the presence or absence of EGF (not shown), although short term EGF treatment induced readily detectable PLCγ phosphorylation in A431 cells. In summary, these results indicated that EGFR activation in primary and HaCaT keratinocytes contributed substantially to MEK/MAPK but not to AKT or STAT3 activation.

Effects of Pharmacological Inhibitors of Signaling Pathways on Keratinocyte Survival—We next examined the effects of small molecular weight compounds commonly used to inhibit signaling through PI 3-kinase/AKT, PLCγ/PKC, and the RAS/RAF/MEK cascade on keratinocyte survival (Table I). We used LY294002 to inhibit PI 3-kinase activity, PD98059 to inhibit MEK-dependent phosphorylation events, the amino steroid U73122 to inhibit PLC-dependent signaling, and Rottlerin and Gö6976 to inhibit PKCδ and PKCα activities, respectively (Table I). In addition, we determined by use of the SRC inhibitors PP1 or PP2 whether SRC family members were required for keratinocyte survival. The inhibitors were used at concentrations needed to inhibit functional activity of the respective signaling pathways. In these assays, the fraction of apoptotic keratinocytes after 24–48-h treatments was determined by TUNEL staining combined with FACS analysis. This assay is

TABLE I
Effects of pharmacological inhibitors of signal transduction pathways on spontaneous apoptosis of normal human keratinocytes (NHK) and immortalized HaCaT keratinocytes

Primary human keratinocytes (NHK) and immortalized HaCaT keratinocytes were seeded in MCDB medium supplemented with insulin (5 μg/ml) and inhibitors at the concentrations indicated. Apoptotic cell death was evaluated by FACS-TUNEL after 48 h; ND, not determined.

Target	Inhibitor (concentration)	TUNEL positive cells	
		NHK	HaCaT
		%	
Control		7	6
EGFR	mAb 425 (66 nM)	8	3
MEK	PD98059 (50 μM)	7	5
PKCδ	Rottlerin (3 μM)	8	12
PI 3-kinase	LY294002 (20 μM)	13	12
SRC	PP1 (20 μM)	17	17
PLC-γ	U73122 (10 μM)	23	ND
PKC-α	Gö6976 (5 μM)	31	30

highly quantitative as it measures the frequency of cells with DNA nicks within at least 2×10^4 cells/sample. We have reported previously (1, 2) that EGFR inhibition by mAb 425 does not induce cell death within the first 24–48 h of treatment but rather sensitizes these cells to cellular stress. To provide a direct comparison between the effects attributable to EGFR blockade and the effects of inhibitors of signal transduction pathways, mAb 425 was included in all assays. As expected, treatment with mAb 425 did not affect survival of either normal keratinocytes or HaCaT cells under steady-state conditions in the absence of cellular stress. Similarly, pharmacological inhibition of MEK or PKCδ activity had only marginable effects on cell survival in this setting. By contrast, inhibition of PI 3-kinase, SRC, PLCγ, and PKCα activities induced significant increases in the fraction of TUNEL-positive cells within 48 h of treatment. The PLCγ inhibitor U73122 and the PKCα inhibitor Gö6976 induced cell death most effectively as judged by the fraction of TUNEL-positive cells after 48 h. Both inhibitors also caused significant cell death as early as 12 h after addition (not shown). These results are consistent with the view that activation of PI 3-kinase, SRC, and PLCγ/PKCα is obligatory to keratinocyte survival but that EGFR activation is not essential to maintain levels of activation of these signaling components required for survival of this cell type.

Effects of Pharmacological Inhibitors of Signaling Pathways on Bcl-x$_L$ Protein Expression—Our previous studies demonstrated that EGFR blockade is associated with down-regulation of Bcl-x$_L$ mRNA and protein expression prior to manifestation
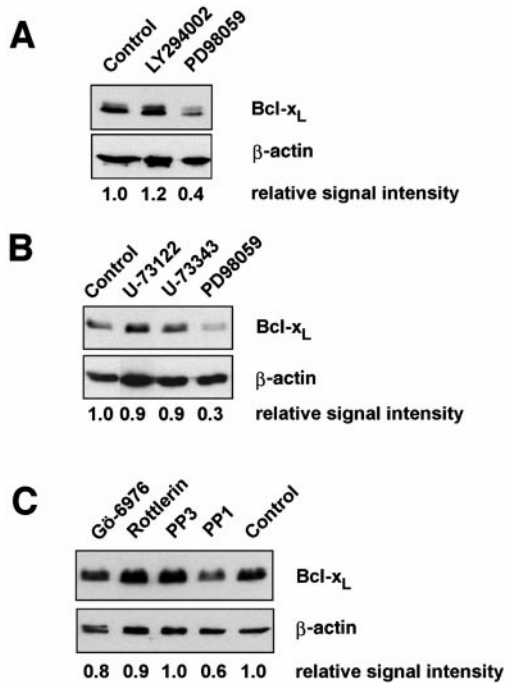
FIG. 2. **Effects of pharmacological inhibitors of signaling pathways on Bcl-x$_L$ protein expression in human keratinocytes.** *A,* effects of MEK inhibitor PD98059 (50 μM) and PI 3-kinase inhibitor LY294002 (20 μM) on Bcl-x$_L$ expression in HaCaT keratinocytes. The blots were reprobed with an β-actin antibody to demonstrate equal loading. *B,* effect of PLCγ inhibitor U73122 (10 μM) on Bcl-x$_L$ expression in normal keratinocytes. Controls consisted of the inactive compound U73343 as well as PD98059. *C,* effect of the SRC inhibitor PP1 (20 μM) on Bcl-x$_L$ expression in HaCaT keratinocytes. Controls consisted of the related but inactive compound PP3 (20 μM). In this experiment Gö6976 (5 μM) and Rottlerin (3 μM) were also used to inhibit PKCα and -δ, respectively. Relative signal intensities represent the ratio of the densitometrically measured Bcl-x$_L$ signal to the β-actin signal in each sample relative to controls shown as 1. Reduction of this ratio >20% was considered significant.

of cell death and that Bcl-x$_L$ overexpression obviates the enhanced apoptosis susceptibility observed in keratinocytes treated with EGFR inhibitors (2, 4). Therefore, we determined the effects of signal transduction inhibitors on Bcl-x$_L$ protein expression as determined by Western blot analysis. These experiments were done after 48 h of incubation with the respective compound. As shown in Fig. 2 and consistent with a very recent report (28), inhibition of either SRC or SRC-like kinases by PP1 was associated with down-regulated Bcl-x$_L$ protein expression in HaCaT cells. Similarly, treatment with the MEK inhibitor PD98059 was associated with consistently lower steady-state Bcl-x$_L$ protein levels. By contrast, inhibition of PI 3-kinase, PLCγ, or PKCα had no detectable effect on Bcl-x$_L$ expression.

Dominant Negative AKT Induces Keratinocyte Apoptosis Independently of Bcl-x$_L$ Expression—To obtain independent evidence for the requirement of PI 3-kinase/AKT signaling for keratinocyte survival, we generated HaCaT cells, which conditionally expressed a dominant negative AKT construct under tetracycline control. This construct encodes an AKT derivative rendered kinase-inactive by point mutation within the AKT catalytic domain (13); upon overexpression in HaCaT cells it was functional as demonstrated by its ability to down-regulate GSK-3β phosphorylation (Fig. 3A). Similar to the PI 3-kinase inhibitor LY294002, overexpressed inactive AKT induced spontaneous keratinocyte apoptosis over a 48-h period (Fig. 3C) but had no effect on Bcl-x$_L$ expression (Fig. 3B) during this time.

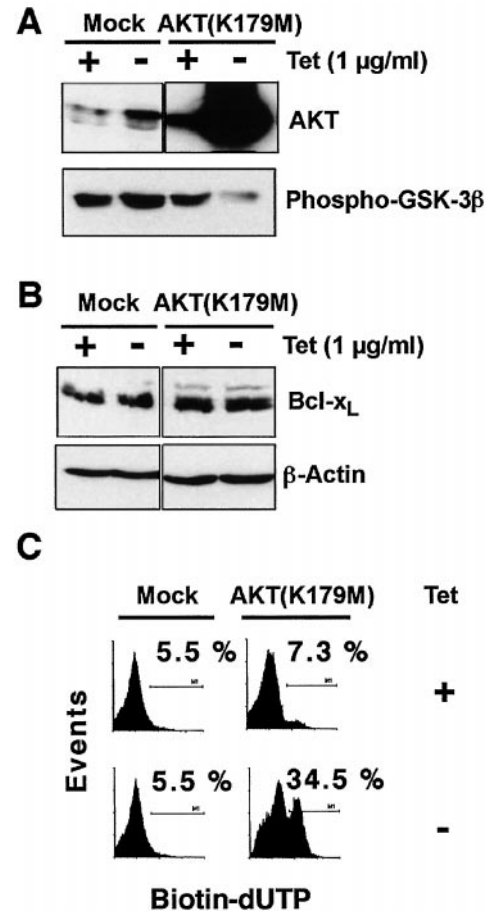*Dominant Negative MEK Down-regulates Bcl-x$_L$ Expression and Impairs Keratinocyte Survival*—To target the MEK/MAPK

FIG. 3. **Induction of apoptosis in HaCaT keratinocytes by conditional expression of a dominant negative AKT construct.** *A,* transgene expression in the presence (+) and absence (−) of tetracycline (*Tet*) and inhibition of GSK-3β phosphorylation in cells overexpressing dominant negative AKT (AKT(K179 M)); cells were maintained in the experimental conditions indicated for 48 h. Mock-transfected HaCaT cells served as controls for tetracycline-induced effects. *B,* expression of Bcl-x$_L$ in HaCaT cells overexpressing dominant negative AKT. *C,* TUNEL staining of HaCaT cells overexpressing dominant negative AKT for 48 h as compared with uninduced cells and mock-transfected controls. The proportion of TUNEL-positive cells in each experimental condition is shown in the respective panels.

pathway specifically, we used a dominant interfering MEK construct that has no kinase activity (11). Upon transfection into HaCaT cells, we first confirmed conditional expression of the MKK1-8E construct (Fig. 4A). When overexpressed, MKK1-8E induced spontaneous apoptosis in HaCaT cells (Fig. 4B) consistent with an important role of MEK-dependent signaling in maintaining keratinocyte survival. Furthermore, overexpression of MKK1-8E was accompanied by moderately but consistently down-regulated Bcl-x$_L$ expression in HaCaT keratinocytes (Fig. 4A); in different experiments Bcl-x$_L$ expression levels were reduced by 30–50% in MKK-8E overexpressing HaCaT keratinocytes when compared with uninduced or mock-transfected HaCaT cells. This result was consistent with the observation that the MEK inhibitor PD98059 reduced Bcl-x$_L$ expression in HaCaT cells (see above). Next, we assessed the effect of transiently transfected MKK1-8E on *Bcl-x* promoter activity. To this end, we used a 1.1-kilobase pair *Bcl-x* promoter fragment driving expression of the luciferase gene in HaCaT keratinocytes (Fig. 5). We observed consistent down-regulation of promoter activity (40–60%) in cells cotransfected with MKK1-8E when compared with cells that were transfected with a mock construct or with HA-AKT(K179M).

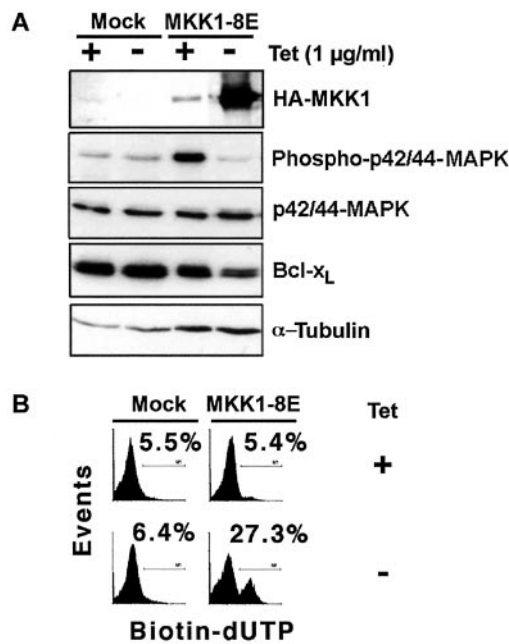*No Effect of Dominant Negative STAT3 on Bcl-x$_L$ Expression*

Signaling Pathways in Keratinocyte Survival

FIG. 4. **Induction of apoptosis in HaCaT keratinocytes by conditional expression of a dominant negative MEK1 construct.** *A,* MKK1-8E transgene expression in the presence (+) and absence (−) of tetracycline after 48 h and inhibition of MAPK phosphorylation in HaCaT cells overexpressing dominant negative MEK. MKK1-8E transgene expression was assessed using an antibody to the hemagglutinin tag. In addition the expression of Bcl-x_L in MKK1-8E-overexpressing cells is shown relative to mock-transfected control cells and to the uninduced state; the blot was reprobed with an antibody to α-tubulin to account for differences in loading between samples. *B,* TUNEL staining of HaCaT cells overexpressing dominant negative MEK as compared with uninduced cells and mock-transfected controls.
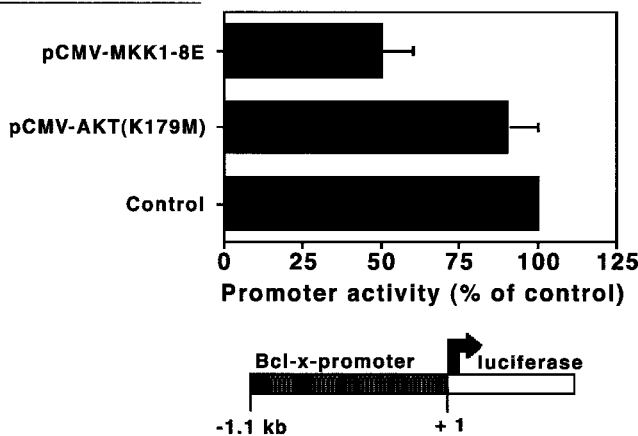

FIG. 5. **Effects of dominant negative MEK on *Bcl-x* promoter activity.** HaCaT cells were transiently cotransfected with MKK1-8E and a *Bcl-x*-luc reporter construct schematically shown at the *bottom* of the figure. Controls included mock-transfected HaCaT cells and cells transfected with the dominant negative AKT construct AKT(K179M).

or Keratinocyte Survival—A recent study identified STAT3 as a critical mediator of EGFR-induced signals in support of Bcl-x_L expression in squamous carcinoma cells (29). Consistent with this idea EGF treatment of A431 squamous carcinoma cells induced STAT3 phosphorylation (see Fig. 1). However, in HaCaT cells, no measurable STAT3 phosphorylation was observed in the absence or the presence of exogenous EGF. This result suggested that STAT3 signaling downstream of the EGFR was not relevant to survival or Bcl-x_L expression in HaCaT cells. To test this contention directly, HaCaT-derived cells were generated in which either one of the two dominant negative STAT3
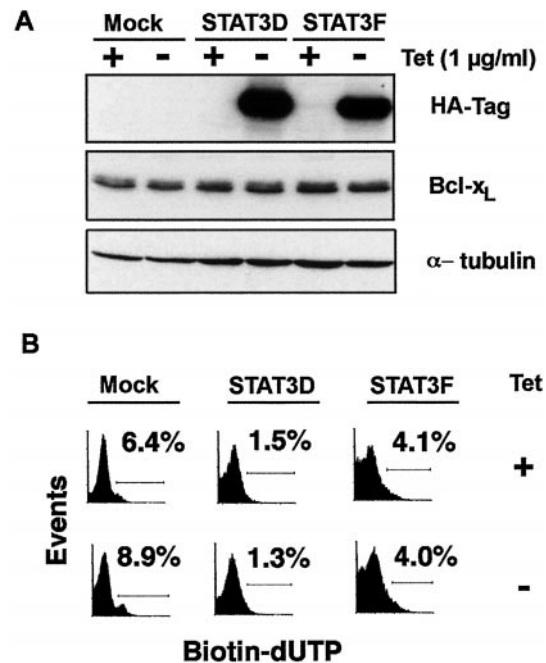

FIG. 6. **Effects of dominant negative STAT3 constructs on Bcl-x_L expression and survival of HaCaT cells.** *A,* expression of the dominant negative STAT3D and STAT3F constructs in HaCaT cells as indicated; transgene expression was probed using an antibody to the hemagglutinin tag. Also shown is Bcl-x_L expression in the presence (+) and absence (−) of tetracycline after 48 h. The blot was reprobed with an antibody to α-tubulin to account for differences in loading between samples. *B,* TUNEL staining of HaCaT cells expressing STAT3D and STAT3F. All experiments were performed 48 h after removal of tetracycline (*Tet*) to induce transgene expression.

constructs (STAT3D and STAT3F) were conditionally expressed using the tetracycline-regulated expression system. As shown in Fig. 6*A,* strong transgene expression was achieved in tetracycline regulatable fashion. However, even after induction of DN STAT3 expression for 2–4 days no significant change in Bcl-x_L protein expression was evident (Fig. 6). Similarly, keratinocyte survival was not affected by overexpression of either of the two dominant negative STAT3 constructs for 2 days (Fig. 6*B*). These results were consistent with the observation that, although expressed in HaCaT keratinocytes, STAT3 plays a minor role in EGFR-induced signal transduction in this cell line (see Fig. 1).

DISCUSSION

The most salient findings of this study can be summarized as follows. 1) Activation of multiple signaling pathways including SRC kinases, MEK1, PI 3-kinase/AKT, and PLCγ/PKCα but not STAT3 were required for keratinocyte survival *in vitro.* 2) EGFR activation in keratinocytes was not necessary to maintain PI 3-kinase or PLCγ/PKCα signaling sufficient to support survival of keratinocytes. 3) By contrast, MEK activity in human keratinocytes was largely dependent on EGFR activation. 4) Inhibition of either the EGFR tyrosine kinase or of MEK enzymatic activity was associated with down-regulation of Bcl-x_L expression and enhanced susceptibility to cell death induction. Based on these results we suggest that EGFR activation supports keratinocyte survival and Bcl-x_L expression in part through activation of the MEK/MAPK cascade (Fig. 7).

Previous work strongly implicates PI 3-kinase in nerve growth factor- and IGF-1-dependent survival of several cell types, including fibroblasts and neuronal cells (13, 30–33) and some hemopoietic cells (34, 35). We extend these observations to human keratinocytes, which similarly required PI 3-kinase/AKT activity for survival. However, our findings also indicate
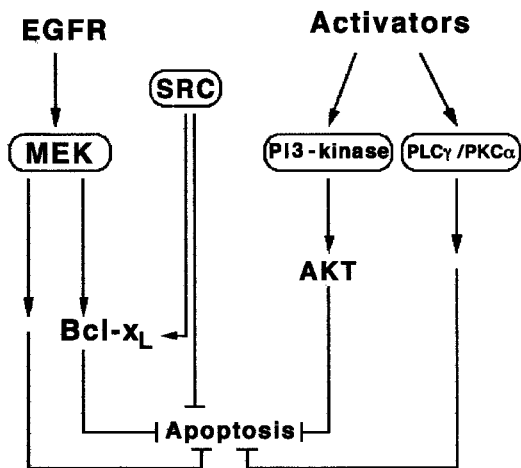
Fig. 7. **Schematic view of signaling pathways required for keratinocyte survival *in vitro*.** Multiple signals depending on MEK, PI 3-kinase, and PLCγ activities appear necessary to sustain survival of this cell type. To maintain MEK activity in cultured keratinocytes EGFR activation is indispensable. By contrast, PI 3-K/AKT and PLCγ/PKCα activation levels required for normal keratinocyte survival can be maintained independently of EGFR activation. MEK-dependent signals regulate expression of Bcl-x$_L$ and, possibly, additional targets relevant to cell survival.

that, in keratinocytes, EGFR activation was not necessary for PI 3-kinase/AKT-dependent protection against apoptosis under steady-state culture conditions. In support of this view, inhibition of the EGFR by use of mAb 425 did not substantially affect AKT-mediated phosphorylation of GSK-3β. This result is consistent with previous observations that the EGFR is relatively ineffective in stimulating PI 3-kinase activity (18, 36). We described earlier that activation of the IGF1R by insulin used at supraphysiological concentrations supports keratinocyte survival under the culture condition used here (4). It remains to be determined whether activation of the IGF-1R contributes to the PI 3-kinase activity necessary for keratinocyte survival as suggested recently (37).

Activation of PLCγ is required for survival of endothelial cells (38) but promotes apoptotic death of myoblasts (39) and thymocytes (40). In hemopoietic precursor cells suppression of PLCγ activity by the inhibitor D609 has been associated with down-regulation of Bcl-2 expression followed by cell death (41). These findings led us to investigate the role of PLCγ in keratinocyte survival and Bcl-x$_L$ expression. We demonstrate that the PLCγ inhibitor U73122 did not affect Bcl-x$_L$ expression in keratinocytes although it effectively induced keratinocyte death. Similar to the PI 3-kinase/AKT pathway, EGFR activation was also not required to sustain basal PLCγ activity. Furthermore, keratinocytes required activation of conventional PKCs (either PKCα, -β, or -γ) but not the Ca^{2+}-independent PKCδ for survival. This conclusion is supported by the observations that the PKCδ inhibitor Rottlerin had no measurable effects on keratinocyte survival, whereas the PKCα inhibitor Gö6976 potently induced keratinocyte apoptosis. These results are consistent with several recent reports in the literature. Specifically, overexpression of PKCδ has been described to induce cell death in keratinocytes (42), and PKCδ participates in caspase-mediated apoptosis in various cell types (43) including human keratinocytes (44). By contrast, PKCα is essential for cell survival in B lymphocytes, glioma (45), and hepatocellular carcinoma cells (46, 47). Furthermore, PKCα overexpression was observed to stimulate AKT activity in a PI 3-kinase-independent manner and suppress apoptosis of 32D myeloid progenitor cells (48). Taken together, these results assign important roles to both PLCγ and PKCα in survival of normal

keratinocytes. However, EGFR activation is not required to maintain PLCγ/PKCα activities sufficient for survival of this cell type in the steady-state culture conditions chosen by us.

In contrast to the PI 3-kinase/AKT and the PLCγ/PKCα pathways, EGFR activation was obligatory to phosphorylation of the MAPK target ELK-1 in keratinocytes. This conclusion is based on the observation that EGFR blockade by use of mAb 425 reduced ELK-1 phosphorylation similar to the MEK inhibitor PD98059. Inhibition of MEK activity by either PD98059 or by use of a dominant interfering MEK construct also down-regulated Bcl-x$_L$ promoter activity and protein expression in a manner similar to EGFR blockade by mAb 425. Induction of the dominant negative MEK construct for 48 h also led to spontaneous apoptosis in the absence of obvious cellular stress indicating that EGFR-independent MEK activity may provide survival signals other than supporting Bcl-x$_L$ expression. Consistent with this view MEK has recently been shown to contribute to phosphorylation of the pro-apoptotic Bcl-2 family member Bad (34, 49). Interestingly, overexpression of the dominant negative MEK1 construct MKK1-8E led to spontaneous apoptosis of HaCaT keratinocytes, whereas pharmacological inhibition of MEK activity did not. At present, this apparent discrepancy is unresolved. It is possible that the dominant negative MEK construct exerts effects on other targets in addition to MEK1 resulting in enhanced cell death.

The results presented here are consistent with an earlier study that implicated both the mitogen-activated protein kinase (MAPK) signaling pathway and SRC family kinases in survival of BaF/3 cells engineered to express the EGFR (50) and establish that the same pathways contribute to Bcl-x$_L$ protein expression in human keratinocytes. Furthermore, our results highlight the requirement for SRC family kinase activity also for keratinocyte survival. SRC can be activated by EGFR engagement in various cell types (51–53) and potentiates the ability of the EGFR to transform murine fibroblasts (54). It remains to be determined whether SRC kinases contribute to Bc-x$_L$ expression in keratinocytes primarily through the MEK/MAPK pathway or through STAT3 activation as described for NIH3T3 cells overexpressing the EGFR (55).

Interestingly, despite robust STAT3 expression we found no evidence for STAT3 phosphorylation in HaCaT cells either in steady-state conditions or when pulsed short term with exogenous EGF. That STAT3 could be phosphorylated by EGFR activation in epithelial cells was shown in A431 squamous carcinoma cells which, however, express very high levels of EGFR. Consistent with a negligible role of STAT3 in regulation of HaCaT cell survival stable overexpression of two different dominant interfering STAT3 constructs in a tetracycline-regulatable manner had no measurable effect on either survival or Bcl-x$_L$ protein expression in these cells. We conclude that, in contrast to AKT, PLCγ, and MEK activation, STAT3-mediated events were not essential to survival of HaCaT keratinocytes. Presently, we are investigating the possibility that STAT3 signaling downstream of the EGFR is a characteristic of advanced epithelial malignancies and does not extend to either normal keratinocytes or early stages of malignant transformation.

In summary, this study provides evidence that interference with several major signal transduction pathways channeled through either AKT, or MEK, or PLCγ/PKCα provoked spontaneous apoptosis of normal and immortalized keratinocytes in culture. These results suggest that keratinocyte survival rests on the coordinate activation of a network of signal transducers and that disruption of any of these pathways will result in death by default. Conversely, disruption of EGF receptor signaling impacted only one, *i.e.* the MEK/MAPK pathway, to the extent that keratinocytes became prone to apoptosis.

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