Brain-derived neurotrophic factor-, epidermal growth factor-, or A-Raf-induced growth of HaCaT keratinocytes requires extracellular signal-regulated kinase

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Rössler, Oliver G., and Gerald Thiel. Brain-derived neurotrophic factor-, epidermal growth factor-, or A-Raf-induced growth of HaCaT keratinocytes requires extracellular signal-regulated kinase. Am J Physiol Cell Physiol 286: C1118–C1129, 2004; 10.1152/ajpcell.00301.2003.—The epidermal growth factor (EGF) receptor plays an important role in epithelial cells by controlling cell proliferation and survival. Keratinocytes also express another class of receptor tyrosine kinases, the neurotrophin receptors. To analyze the biological role of the neurotrophin brain-derived neurotrophic factor (BDNF) in keratinocytes, we expressed the BDNF receptor TrkB in immortalized human HaCaT keratinocytes. Stimulation of HaCaT-TrkB cells with BDNF induced DNA synthesis and increased mitochondrial reduction capacities, both indications of proliferating cells. An analysis of the signal transduction cascade revealed that the activated TrkB receptor effectively utilized components of the EGF receptor signaling pathway to control cell proliferation. Mitogenic signaling induced by BDNF or EGF was completely abrogated by the MAP kinase kinase (MEK) inhibitor PD-98059, whereas inhibition of phosphatidylinositol 3-kinase (PI3-kinase), activation of signal transducers and activators of transcription (STAT), and activation of phosphatidylidylinositol 3-kinase (PI3-kinase), activation of signal transducers and activators of transcription (STAT), and activation of phospholipase C-γ. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The results presented in this study show that EGF and BDNF function as potent mitogens for HaCaT keratinocytes, and this
activity depends on the activation of ERK. The crucial role of ERK was further demonstrated in HaCaT cells expressing a conditionally active form of A-Raf. Finally, the results show that EGF and BDNF, as well as activation of A-Raf, rapidly induced biosynthesis of the transcription factors Egr-1 and c-Jun.

MATERIALS AND METHODS

Cell culture. The immortalized human cell line HaCaT was a kind gift of N. E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37°C in 5% CO₂. Cells were stimulated with BDNF (catalog no. G1491, Promega, Mannheim, Germany; final concentration 1–20 ng/ml, diluted in PBS containing 0.1% bovine serum albumin) or EGF (catalog no. G5021, Promega; final concentration 0.5–10 ng/ml, dissolved in PBS) for 1–20 min. The MAP kinase kinase (MEK) inhibitor PD-98059 (catalog no. S13000, Calbiochem, Darmstadt, Germany) was dissolved in DMSO and used at 50 μM. Wortmannin (catalog no. 681675, Calbiochem) was dissolved in DMSO and used at 1 μM. Cells were preincubated with PD-98059 or wortmannin for 6 h. 4-Hydroxytamoxifen (4-OHT) was purchased from Sigma (catalog no. H7904) and used at 25 mM, with ethanol used as the solvent.

Retroviral gene transfer. The retroviral vector pMSCVpac (17) was a kind gift of R. G. Hawley (Sunnybrook Health Science Centre, Toronto, ON, Canada). Plasmid TrkB.TK(+)Flag-pEF/BOS (16), encoding FLAG-tagged TrkB receptor, was a kind gift of A. Haapasalo and E. Castrén (A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland). The coding region for the FLAG-tagged TrkB receptor was excised with BamHI and HpaI and cloned into the BglII/HpaI sites of pMSCVpac, generating the retroviral expression plasmid pMSCV-FLAG-TrkB.TK. Plasmid pBabepuro3αAraf:ER, encoding an activated form of the protein kinase A-Raf as a fusion protein with the hormone binding domain of the murine estrogen receptor (ER™, estrogen receptor tamoxifen mutant), was kindly provided by M. McMahon (Cancer Research Institute and Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA) (29). The packaging cell line Phoenix-Ampho was obtained from G. Nolan (Stanford University, Palo Alto, CA). Cells were transfected with retroviral vectors according to the protocol developed by G. Nolan (www.stanford.edu/group/nolan/NL-helper.html) using the calcium coprecipitation procedure. Viral supernatants were harvested 72 h after transfection, filtered through a 0.45-μm filter, and used to infect HaCaT cells in the presence of 8 μg/ml polybrene at 37°C. After 48 h, the medium was removed, and the cells were supplied with fresh complete medium and cultured for 72 h before addition of selection medium containing 0.6 μg puromycin/ml. Mass pools of stable transfectants were selected and used for all experiments to eliminate the possibility of specific clonal effects.

Proliferation assays. Cells were seeded in 96-well plates at a density of 3 × 10³ cells/well and incubated for 24 h. The serum concentration was lowered to 0.05%, and the cells were incubated for another 24 h. The cells were stimulated with BDNF and EGF for 24 or 48 h and incubated with 4-OHT for 48 h. Induction of DNA synthesis was measured by incorporation of the pyrimidine analog 5-bromo-2'-deoxyuridine (BrdU), instead of thymidine, into the DNA of proliferating cells using the cell proliferation ELISA kit (catalog no. 1647229, Roche Diagnostics, Mannheim, Germany). The assay was performed according to the instruction manual with minor modifications. The labeling time with BrdU was 2 h, and incubation with the anti-BrdU peroxidase antibody was 90 min. Peroxidase activity was determined spectrophotometrically as described in the instruction manual. Each experiment was performed in quadruplicate, and the mean ± SD is depicted.

The mitochondrial reduction capacities were determined by quantification of the level of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) reduction to formazan dye crystals (MTT assay). HaCaT cells were plated in quadruplicate in 96-well plates at a density of 3 × 10⁴ cells/well and incubated for 24 h. The serum concentration was reduced to 0.05% after 24 h, cells were stimulated with BDNF, EGF, 4-OHT, or vehicle for 48 h. MTT solution (0.5 mg/ml final concentration/well, dissolved in PBS) was added to the cultures, which were then incubated for 4 h at 37°C in 5% CO₂. Crystals were solubilized in 10 mM HCl containing 10% SDS, and the plates were incubated overnight at 37°C. Absorbance was quantified on a microplate reader (model 550, Bio-Rad) using a test wavelength of 595 nm. MTT reduction was expressed as a percentage of controls. All experiments represent at least two independent replications performed in quadruplicate.

Transient transfections and reporter gene assays. Plasmids pColl(−517/+63)luc and pEBS1luc have been described elsewhere (6, 43). Plasmid pColl(−517/+63)luc contains the human collagenase regulatory sequence from −517 to +63 upstream of the luciferase open reading frame. The minimal Egr-1-responsive reporter plasmid pEBS1luc contains four binding sites for Egr-1 derived from the Egr-1 promoter upstream of a minimal promoter consisting of the human immunodeficiency virus TATA box and the adenovirus major late promoter initiator element. The expression vector of murine Egr-1, pCMV-Egr-1, formerly termed pCMVzif, has been described elsewhere (44).

HaCaT cells were seeded at a density of 5 × 10³ cells/plate onto 35-mm plates. Cells were transfected using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s protocol. FuGENE 6 was diluted with DMEM and mixed with DNA in the ratio 1 μg of DNA to 3 μl of FuGENE 6. The mixture was incubated for 45 min at room temperature and then added to the cells and incubated for 24 or 48 h in medium containing 10% serum. The serum concentration was lowered to 0.05%, and the cells were incubated for a further 24 h. Cells were stimulated with EGF and BDNF for 6 h and with 4-OHT for 16 h. Transfection experiments involving expression vectors encoding Egr-1 or C2/c-Jun were performed in the presence of pRSVβ (0.8 μg/plate) to correct for variations in the transfection efficiencies. Cell extracts were prepared and reporter gene activity was determined as described elsewhere (43), except chlorphenol red-β-galactosidase (Roche Molecular Biochemicals) was used as a substrate for β-galactosidase.

Preparation of cell extracts. Whole cell extracts, nuclear extracts, and crude membranes were prepared as described elsewhere (22).

Antibodies and immunoblot analysis. To detect the phosphorylated form of ERK or c-Akt, 50 μg of proteins derived from whole cell extract preparations were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed with an antibody directed against ERK (catalog no. sc-153, Santa Cruz Biotechnology, Heidelberg, Germany), the phosphorylated form of ERK (catalog no. V8031, Promega), or a mixture of two antibodies directed against the phosphorylated residues Ser173 and Thr326 of c-Akt (catalog nos. 9271 and 9275, New England Biolabs). To analyze Egr-1 synthesis, 20 μg of nuclear proteins were separated by 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The blots were incubated with antibodies directed against human Egr-1 (catalog no. sc-110, Santa Cruz Biotechnology) or human c-Jun (catalog no. sc-1694, Santa Cruz Biotechnology). To analyze TrkB receptor expression, 10 μg of proteins from a crude membrane preparation were separated by a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The blot was incubated with M2 monoclonal antibody (catalog no. F3165, Sigma) directed against the FLAG epitope present at the NH2 terminus of the expressed TrkB receptor. Blots were developed using horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (catalog nos. 111-035-003 and 115-035-003, Dianova, Hamburg, Ger-
many) and enhanced chemiluminescence (Amersham, Freiburg, Germany).

Statistical analysis. P values were determined using one-way ANOVA.

RESULTS

Generation of HaCaT cells expressing the TrkB neurotrophin receptor. The TrkB neurotrophin receptor belongs to the superfamily of receptors with intrinsic tyrosine kinase activity. The modular structure of TrkB is depicted in Fig. 1A, which shows the extracellular domain, a single transmembrane domain, and the cytoplasmic portion of the receptor, including the tyrosine kinase domain. To investigate the functional role of BDNF in keratinocytes and to compare the biological outcome of EGF and BDNF stimulation of keratinocytes, we used immortalized human HaCaT keratinocytes as a model system. We infected HaCaT cells with a recombinant retrovirus encoding a FLAG-tagged TrkB receptor under control of the murine stem cell virus long terminal repeat. The location of the FLAG tag is indicated in Fig. 1A. As a control, HaCaT cells were infected with recombinant retroviruses encoding puromycin acetyltransferase (HaCaTpac cells). The resultant keratinocyte cell line HaCaT-TrkB served two purposes: 1) given the fact that wild-type HaCaT cells do not express functional TrkB receptors, we were able to analyze BDNF/TrkB signaling in a heterologous cellular system; and 2) the reports describing TrkB expression in keratinocytes indicate that BDNF functions as a physiological ligand in keratinocytes. Thus, if HaCaT-TrkB cells responded to BDNF in a similar manner to primary keratinocytes, the cells could be used as a model cell line to study the cell biology of BDNF in epithelial cells. Expression of the TrkB receptor was tested in Western blot experiments using antibodies specific for the FLAG epitope. Figure 1B shows that the FLAG-tagged TrkB receptor could be immunologically detected in HaCaT-TrkB cells but not in HaCaTpac control cells.

BDNF and EGF activate phosphorylation of ERK and c-Akt in HaCaT-TrkB and HaCaTpac cells. Activation of the EGF receptor triggers the phosphorylation and activation of the protein kinases ERK1 and ERK2 in HaCaT cells (22). We used an antibody directed against the phosphorylated p42 isoform of ERK, termed ERK2, to analyze the effect of BDNF stimulation of HaCaT-TrkB cells on the activation state of ERK. Administration of BDNF induced phosphorylation, i.e., activation of ERK2, in HaCaT-TrkB cells (Fig. 2A). Similarly, EGF induced an activation of ERK in HaCaTpac cells (Fig. 2B, top). BDNF- and EGF-triggered phosphorylation of ERK was completely blocked by preincubation of the cells with PD-98059 (Fig. 2, A and B, right lanes). This compound inhibits phosphorylation of MEK, thus blocking the activation of ERK (10). No activation of ERK was observed in BDNF-stimulated HaCaTpac cells that lacked TrkB receptor expression (Fig. 2B, bottom). Activation of ERK by BDNF or EGF was robust but transient (Fig. 2C). Both ligands stimulated a rapid phosphorylation of ERK. Within 15 min after stimulation, phosphorylated ERK could be detected. In BDNF-treated HaCaT-TrkB cells, ERK remained phosphorylated for ≥1 h. In EGF-treated HaCaTpac cells, ERK was already dephosphorylated and inactivated 1 h after stimulation.

The PI3-kinase pathway can be directly activated by recruitment to tyrosine-phosphorylated receptors or, indirectly, through an activated Ras. Active PI3-kinase catalyzes the synthesis of 3'-phosphorylated inositol lipids, which control the intracellular localization and activity of a key molecule of PI3-kinase signaling, the protein kinase c-Akt. Phosphorylation of c-Akt serves as an indicator for a previous PI3-kinase activation. Incubation of HaCaT-TrkB cells with BDNF or EGF induced the phosphorylation and activation of c-Akt, as detected with phosphospecific antibodies (Fig. 2D, left lanes). BDNF- and EGF-triggered c-Akt phosphorylation was prevented by preincubation of the cells with the PI3-kinase inhibitor wortmannin (Fig. 2D, right lanes). Wortmannin also caused a slight reduction of EGF- or BDNF-triggered ERK activation (Fig. 2E). Taken together, activation of the EGF or BDNF receptor signaling pathways leads to the phosphorylation and activation of the protein kinases ERK and c-Akt.

BDNF and EGF stimulate biosynthesis of the transcription factors Egr-1 and c-Jun in HaCaT-TrkB and HaCaTpac cells. To follow the BDNF and EGF signaling cascade in HaCaT-TrkB and HaCaTpac cells, we analyzed the expression of the transcription factors Egr-1 and c-Jun. Transcription of the Egr-1 gene has been shown to be induced by stimulation with EGF or neurotrophins (14, 22, 30). Similarly, c-Jun gene expression has been reported to be controlled by growth factors...
To test the effects of BDNF and EGF on Egr-1 and c-Jun biosynthesis, HaCaT-TrkB and HaCaTpac cells were serum starved for 24 h and then incubated with BDNF or EGF for 15 min, or left untreated (−). The effect of the MAP kinase inhibitor PD-98059 on activity of ERK protein kinase was investigated in HaCaT-TrkB cells that had been preincubated for 6 h with PD-98059. B: HaCaTpac cells were serum starved for 24 h and treated with EGF (0.5 or 1 ng/ml, top) or with BDNF (20 ng/ml, bottom) for 15 min. In addition, HaCaTpac cells were preincubated for 6 h with PD-98059 before stimulation with EGF. The effect of PD-98059 on ERK phosphorylation is indicated. C: time course of BDNF- or EGF-induced ERK activation. HaCaT-TrkB cells (top) or HaCaTpac cells (bottom) were serum starved for 24 h and then treated with BDNF (5 ng/ml, top) or EGF (1 ng/ml, bottom). Whole cell extracts were prepared and subjected to Western blot analysis. Blots were incubated with an affinity-purified rabbit antibody directed against the phosphorylated active form pp42 (ERK2).

Fig. 2. Phosphorylation and activation of extracellular signal-regulated protein kinase (ERK) and phosphatidylinositol 3-kinase (PI3-kinase) in HaCaT-TrkB and HaCaTpac cells after stimulation with brain-derived neurotrophic factor (BDNF) or epidermal growth factor (EGF). A: HaCaT-TrkB cells were serum starved for 24 h, treated with BDNF (1 or 5 ng/ml) for 15 min, or left untreated (−). The effect of the MAP kinase kinase (MEK) inhibitor PD-98059 on activity of ERK protein kinase was investigated in HaCaT-TrkB cells that had been preincubated for 6 h with PD-98059. B: HaCaTpac cells were serum starved for 24 h and treated with EGF (0.5 or 1 ng/ml, top) or with BDNF (20 ng/ml, bottom) for 15 min. In addition, HaCaTpac cells were preincubated for 6 h with PD-98059 before stimulation with EGF. The effect of PD-98059 on ERK phosphorylation is indicated. C: time course of BDNF- or EGF-induced ERK activation. HaCaT-TrkB cells (top) or HaCaTpac cells (bottom) were serum starved for 24 h and then treated with BDNF (5 ng/ml, top) or EGF (1 ng/ml, bottom). Whole cell extracts were prepared and subjected to Western blot analysis. Blots were incubated with an affinity-purified rabbit antibody directed against the phosphorylated active form pp42 (ERK2). D: phosphorylation and activation of c-Akt in BDNF- and EGF-treated HaCaT-TrkB cells. HaCaT-TrkB cells were serum starved for 24 h and then treated with BDNF (20 ng/ml) or EGF (10 ng/ml) for 15 min or left untreated. The effect of the PI3-kinase inhibitor wortmannin on c-Akt phosphorylation was investigated in HaCaT-TrkB cells that had been preincubated for 6 h with 1 μM wortmannin. E: role of the PI3-kinase inhibitor wortmannin on EGF- and BDNF-induced activation of ERK. HaCaT-TrkB cells were serum starved for 24 h and then treated with EGF or BDNF (1 or 5 ng/ml) for 15 min in the presence or absence of 1 μM wortmannin. Blots were incubated with antibodies directed against ERK or the phosphorylated active form pp42 (ERK2).

(15, 28). To test the effects of BDNF and EGF on Egr-1 and c-Jun biosynthesis, HaCaT-TrkB and HaCaTpac cells were serum starved for 24 h and then incubated with BDNF or EGF for 15 min, 1 h, 4 h, or 8 h. The cells were harvested, and nuclear extracts were prepared and analyzed by Western blotting using antibodies directed against Egr-1 or c-Jun. BDNF (Fig. 3A) and EGF (Fig. 3B) strikingly increased the biosynthesis of Egr-1, with a peak of expression at 1 h after stimulation. An enhancement of c-Jun biosynthesis was also observed, with high levels of c-Jun immunoreactivity detectable 1 h after stimulation. However, the induction was not as strong as that observed for Egr-1 and lasted longer. The results show that stimulation of HaCaTpac cells with EGF or of HaCaT-TrkB cells with BDNF induced Egr-1 biosynthesis with very similar kinetics. Likewise, c-Jun synthesis was activated very similarly by EGF or BDNF in HaCaTpac and HaCaT-TrkB cells, respectively. Thus, in terms of phosphorylation and activation of ERK and stimulation of Egr-1 and c-Jun biosynthesis, the signaling cascades initiated by the BDNF/TrkB or EGF/EGF receptor system are largely comparable.

EGF and BDNF increase the transcriptional activation potential of Egr-1 and c-Jun in HaCaT cells. The ability of Egr-1 to activate transcription depends on the concentrations of the Egr-1 negative cofactors NAB1 and NAB2. These proteins bind to Egr-1 and block transcriptional activation via Egr-1 (36, 41, 43). Thus elevated Egr-1 protein levels do not auto-
BDNF and EGF induce proliferation of HaCaT cells expressing their cognate receptors. The fact that the signaling pathway initiated via the neurotrophin BDNF/TrkB system is very similar to that induced by EGF led us to analyze the mitogenic activity of BDNF in HaCaT-TrkB cells. As a molecular indicator for proliferation, DNA synthesis was measured by incorporation of BrdU into the DNA. Incorporation of BrdU was used as a measure of DNA replication and served as an indicator of cellular activity in the S phase of the cell cycle. HaCaT-TrkB cells were serum starved for 24 h and then treated with BDNF or EGF for 24 h. Figure 5A, left, shows that EGF and BDNF induced a significant increase in BrdU incorporation in HaCaT-TrkB cells, indicating that EGF and BDNF function as mitogens for these cells. In contrast, BDNF did not show any effect on cell proliferation in HaCaTpac cells that did not express TrkB neurotrophin receptors (Fig. 5B, left). We repeated these experiments using the reduction of tetrazolium salts by mitochondrial NAD(P)H-dependent dehydrogenases to formazan (MTT assay). Here, the overall metabolic activity of the cells is measured and used as an indirect indicator of the viable cell number. Previously, neurotrophin-induced cell growth had been measured with the MTT assay (18). HaCaT-TrkB cells were serum starved for 24 h and then treated with BDNF or EGF for 48 h, and cell growth was measured by the MTT assay. The results of the MTT assay are compared with the amount of formazan formed in the absence of EGF or BDNF. Figure 5A, right, shows that EGF and BDNF induced a significant increase in the mitochondrial reduction capacities. BDNF did not show any effect in TrkB-lacking HaCaTpac cells (Fig. 5B, right). EGF, however, was a potent mitogen for HaCaT-TrkB and HaCaTpac cells, both expressing functional EGF receptors.

To confirm the role of ERK or PI3-kinase in EGF- and BDNF-induced cell proliferation, we preincubated HaCaT-TrkB cells with the MEK inhibitor PD-98059 or the PI3-kinase inhibitor wortmannin before stimulating the cells with EGF or BDNF. DNA synthesis was measured by incorporation of BrdU into DNA 24 or 40 h after stimulation. Figure 6, A and B, middle, shows that PD-98059 efficiently blocked BrdU incorporation into DNA in HaCaT-TrkB cells that had been stimulated with EGF or BDNF. PD-98059 did not significantly affect cell viability (data not shown), in agreement with published data (20). The PI3-kinase inhibitor wortmannin delayed the DNA synthesis of EGF- or BDNF-stimulated HaCaT-TrkB cells (Fig. 6, A and B, right) but was unable to finally block the proliferation of the cells. These data were confirmed using the MTT assay (Fig. 6C). Taken together, these results indicate that the mitogenic activity of EGF and BDNF is mediated by an activated ERK, and not by activation of the PI3-kinase pathway.

Conditional activation of the ERK signaling pathway in human HaCaT keratinocytes by expression of a ΔA-Raf:ER fusion protein. In the previously described experiments, we distinguished between the receptor tyrosine kinase-induced activation of ERK or PI3-kinase pathway using pharmacological inhibitors. To specifically activate the ERK pathway in HaCaT cells and to confirm the importance of the ERK signaling pathway for HaCaT cell proliferation, we generated HaCaT cells expressing a ΔA-Raf:ER fusion protein. The modular structure of the Raf protein kinase is depicted in Fig. 7A. The A-Raf protein kinase contains three domains: CR1,
CR2 and CR3. CR1 is a cysteine-rich region and functions as a binding site for activated Ras-GTP at the cell membrane. CR2 is rich in serine and threonine residues and negatively regulates the biological activity of the catalytic domain, perhaps by direct protein-protein interaction with the kinase domain. CR3 encompasses the protein kinase domain. Expression of this catalytic domain of A-Raf as a fusion protein with the ligand binding domain of the murine ER keeps the protein kinase active. CR1 is a cysteine-rich region and functions as a binding site for activated Ras-GTP at the cell membrane. CR2 is rich in serine and threonine residues and negatively regulates the biological activity of the catalytic domain, perhaps by direct protein-protein interaction with the kinase domain. CR3 encompasses the protein kinase domain. Expression of this catalytic domain of A-Raf as a fusion protein with the ligand binding domain of the murine ER keeps the protein kinase active. CR1 is a cysteine-rich region and functions as a binding site for activated Ras-GTP at the cell membrane.

Fig. 4. EGF and BDNF increase transcriptional activation potential of Egr-1 and c-Jun in human HaCaT keratinocytes. A: reporter plasmid pEBS1\(^{\text{TM}}\)luc containing the luciferase reporter gene and a TATA box upstream of which 4 copies of the Egr-1 binding site derived from the Egr-1 promoter termed EBS were inserted. B: reporter plasmid pEBS1\(^{\text{TM}}\)luc (0.2 \(\mu\)g/plate) was transfected into HaCaT cells together with the pRSV plasmid internal standard plasmid (0.8 \(\mu\)g/plate) and an expression vector encoding Egr-1 (pCMVEgr-1, 0.4 \(\mu\)g/plate). Cells were incubated for 24 h with medium containing 10% serum. Serum concentration was lowered to 0.05%, and cells were incubated for 24 h. Values are means ± SD. C: HaCaT cells were transfected with the reporter plasmid pEBS1\(^{\text{TM}}\)luc (0.8 \(\mu\)g/plate). Cells were incubated with medium containing 10% serum for 48 h. Serum concentration was changed to 0.05%, and cells were incubated for 24 h. Cells were stimulated with EGF (1 ng/ml) or BDNF (5 ng/ml) for 6 h. Values are means ± SE. **Statistically significantly different from control (P < 0.005). D: reporter plasmid pColl(−517/+63)luc, containing regulatory regions of the collagenase gene linked to the luciferase coding region. TRE, 12-O-tetradecanoylphorbol-13-acetate response element. E: HaCaT cells were transfected with the reporter plasmid pColl(−517/+63)luc reporter plasmid (0.2 \(\mu\)g/plate), the reference plasmid pRSV \(\beta\) (0.8 \(\mu\)g/plate), and an expression vector encoding a constitutively active CREB2/c-Jun mutant (0.4 \(\mu\)g/plate). F: HaCaT keratinocytes were transfected with the reporter plasmid pColl(−517/+63)luc (0.8 \(\mu\)g/plate). Cells were incubated with medium containing 10% serum for 48 h. Serum concentration was lowered to 0.05%, and cells were incubated for 24 h. Cells were stimulated with EGF (1 ng/ml) or BDNF (5 ng/ml) for 8 h. Values are means ± SD. **Statistically significantly different from control (P < 0.005).
of the catalytic function of A-Raf induced DNA synthesis in HaCaT-\(\Delta A\)-Raf:ER cells. In contrast, 4-OHT was without effect on BrdU incorporation in HaCaTpac cells, which express puromycin acetyltransferase, instead of the conditionally activated form of A-Raf (Fig. 8B, left). We confirmed these data with the MTT assay as a measure for the number of viable cells. HaCaT-\(\Delta A\)-Raf:ER cells were starved for 24 h and then incubated with 4-OHT for 48 h. The mitochondrial reduction capacities were determined and are depicted in comparison with untreated cells. Figure 8, A and B, right, shows that activation of the catalytic function of A-Raf increased the mitochondrial reduction capacities of HaCaT-\(\Delta A\)-Raf:ER, but not HaCaTpac, cells.

The relevance of ERK activation for \(\Delta A\)-Raf:ER-mediated proliferation was studied with the MEK inhibitor PD-98059. The cells were preincubated with PD-98059 and then incubated with 4-OHT for 48 h. DNA synthesis was measured by incorporation of BrdU into DNA. Figure 8C, left, shows that PD-98059 efficiently blocked BrdU incorporation into DNA of HaCaT-\(\Delta A\)-Raf:ER cells that had been stimulated with 4-OHT. Moreover, the increase of the number of viable cells by activation of \(\Delta A\)-Raf:ER was also dependent on the ERK signaling pathway, as shown by the lack of stimulation by 4-OHT in the presence of the MEK inhibitor PD-98059 (Fig. 8C, right). In contrast, we were unable to impair proliferation with the PI3-kinase inhibitor wortmannin (Fig. 8D). These data indicate that the mitogenic activity of \(\Delta A\)-Raf:ER is mediated by the sustained activation of ERK.

**DISCUSSION**

Cell growth of HaCaT keratinocytes is stimulated by EGF and thrombin, which trigger an activation of the EGF receptor (22). These observations are in agreement with the belief that the EGF receptor plays an essential role in promoting cell survival and proliferation and antagonizing cell death in keratinocytes (20, 21, 34, 39). In addition to this widely documented role of EGF and EGF receptor activation in growth control of keratinocytes, the physiological role of the neurotrophin BDNF has also been linked to keratinocyte proliferation. An increased epidermal thickness and an increased number of proliferating keratinocytes in the epidermis have been observed in transgenic mice overexpressing BDNF compared with wild-type mice. Likewise, BDNF-lacking transgenic mice showed a significantly reduced number of proliferating epidermal keratinocytes (2). The objective of this study was to analyze the cellular events triggered by BDNF stimulation in human keratinocytes expressing the BDNF receptor TrkB. Moreover, we compared the signaling cascades and the final physiological alterations induced by EGF and BDNF in keratinocytes.

The generation of human HaCaT keratinocytes expressing the TrkB neurotrophin receptor is reported here. We wanted to analyze the effect of BDNF in a keratinocyte cell line that does not express the cognate TrkB neurotrophin receptor. Similar experiments have been done with fibroblasts expressing the TrkA or TrkB neurotrophin receptor. These fibroblasts, engineered to express the neurotrophin receptors TrkA or TrkB, proliferated as a result of stimulation with their cognate ligands BDNF and nerve growth factor (NGF) (12). However, a growth-inhibitory activity of NGF on TrkA-expressing NIH...
3T3 fibroblasts was also described (9). Our results show that BDNF functions as a mitogen for TrkB-expressing HaCaT keratinocytes. Thus HaCaT-TrkB cells show a very similar physiological response to BDNF compared with primary keratinocytes. This indicates that we have generated a cellular model that may be very valuable as an easily accessible system to study the cell biology of BDNF in a keratinocyte environment. The creation and validation of this model system represent the second objective of our work. HaCaT cells have been described to exhibit deficiencies in organotypic cocultures with fibroblasts, representing an in vitro skin equivalent model, although essentially all epidermal differentiation markers are expressed. A recent study showed that supplementation of HaCaT cells with transforming growth factor-α (TGF-α), an

Fig. 6. Effects of MEK and PI3-kinase inhibitors on BDNF- or EGF-induced proliferation of HaCaT-TrkB cells. HaCaT-TrkB cells were cultured as described in Fig. 5 legend and stimulated with EGF (1 ng/ml) or BDNF (5 ng/ml). Cells were preincubated with vehicle (left), 50 μM PD-98059, or 1 μM wortmannin for 6 h. Incorporation of BrdU into the DNA was measured 24 (A) or 40 h (B) after stimulation. C: results of MTT assay performed 48 h after stimulation with BDNF or EGF. Values are means ± SD of quadruplicate wells from a single typical experiment. **Statistically significantly different from control (P < 0.005).
EGF receptor ligand, restored the capacity of the cells to form structured epithelia in organotypic cocultures (27). TGF-β/H9251 enhanced expression of interleukin-1 and the receptors for keratinocyte growth factor and granulocyte-macrophage-colony-stimulating growth factor in HaCaT cells, thereby restoring the delayed and deficient growth and differentiation capacities of the cells. Epidermal tissue differentiation of TGF-β/H9251-stimulated HaCaT cells was shown to be comparable to cultures of normal human skin keratinocytes (27), indicating that TGF-β/H9251-stimulated HaCaT cells are a regular skin equivalent that may be very useful as a highly standardized in vitro tissue model.

The signaling pathways and the biological effects induced by BDNF in HaCaT-TrkB cells or by EGF in HaCaT or HaCaTpac cells were shown to be very similar, indicating that activation of the TrkB or the EGF receptor tyrosine kinase function is connected with the same or a very similar signaling cascade in keratinocytes. The fact that supplementation of HaCaT cells with EGF also normalized regeneration of epidermal tissue differentiation suggests that BDNF stimulation of HaCaT-TrkB cells may also be sufficient to restore the capacity of the cells to form structured epithelia in organotypic cocultures. Activation of the ERK signaling pathway by Ras and Raf and activation of PI3-kinase have been described to be essential for the induction of cell growth of epithelial cells. Here, we have shown that the activation of ERK is crucial for EGF- and BDNF-mediated the induction of HaCaT keratinocyte proliferation. These assumptions are based on experiments performed with the MEK inhibitor PD-98059, which prevents activation of this kinase by the “upstream” protein kinase Raf. PD-98059 has a very impressive selectivity profile, as demonstrated by the fact that no other protein kinase was inhibited by this compound when used at a concentration of 50 μM (8). PD-98059 inhibited EGF- or BDNF-induced cell proliferation in HaCaT-TrkB cells, indicating that activation of ERK is required for the growth-promoting activity of both ligands.

The biological role of the ERK signaling pathway in the control of keratinocyte proliferation was further investigated using a conditionally active form of A-Raf. Analysis of the signaling cascade induced by activation of A-Raf showed that the kinetics of ERK activation (transient vs. sustained) are of less importance for the induction of the mitogenic program of keratinocytes.

Fig. 7. Modular structure and signaling of ΔA-Raf:ER, a conditionally active form of the A-Raf protein kinase. A: modular structure of A-Raf and ΔA-Raf:ER. Functional domains of A-Raf, CR3, CR2, and CR1, are depicted. Fusion of the catalytic CR3 domain to the hormone binding domain of the estrogen receptor tamoxifen mutant (ERTM) generates the ΔA-Raf:ER fusion protein. B and C: HaCaT-ΔA-Raf:ER cells were serum-starved for 24 h, treated with 25 nM 4-hydroxytamoxifen (4-OHT), or left untreated (−). Whole cell extracts (B) and nuclear extracts (C) were prepared from different time points and subjected to Western blot analysis. Blots were incubated with an affinity-purified rabbit antibody directed against the phosphorylated active form of ERK2 (B) or with an antisera directed against Egr-1 (C, top) or c-Jun (C, bottom). D and E: HaCaT-ΔA-Raf:ER cells were transfected with the reporter plasmids pEBS1°luc and pColl(−517/+63)luc. Cells were incubated for 48 h with medium containing 10% serum. Serum concentration was changed to 0.05%, and cells were incubated for 24 h. Cells were stimulated with 4-OHT for 16 h. Values are means ± SD. Statistically significantly different from control: *P < 0.05; **P < 0.005.
This observation is in contrast to the role of ERK in neuronal survival, where a sustained activation of ERK is required for neuroprotection (35). Similarly, neuronal differentiation of PC12 cells has been connected with a sustained activation of ERK (46).

Activation of PI3-kinase has been linked to many key cellular functions in mammalian cells, including cell survival and cell proliferation. In Mv1Lu mink lung epithelial cells, for example, PI3-kinase activity has been shown to be essential for hepatocyte growth factor-induced mitogenic signals (32). Likewise, insulin-like growth factor I-induced DNA synthesis and cell division of human breast cancer cells were blocked by the PI3-kinase inhibitor LY-294002, but not by PD-98059, indicating that mitogenic signaling of these cells requires PI3-kinase and is independent of ERK (11). The results described in this study reveal that EGF-, BDNF-, and A-Raf-induced cell growth uses the ERK signaling pathway, independent of PI3-kinase activation. The PI3-kinase inhibitor wortmannin delayed the mitogenic response to EGF or BDNF, but 40 h after stimulation with the growth factors, similar proliferation rates were measured in the presence or absence of wortmannin. The delay of EGF- or BDNF-induced DNA synthesis observed after treatment of the cells with wortmannin may be due to the slight reduction of ERK activation. Thus wortmannin did not block EGF- or BDNF-induced activation of ERK but reduced the levels of active ERK (Fig. 2E). Similar observations have
been reported for CCl39 fibroblasts and COS cells (5, 45). How wortmannin reduces ERK activation is not yet clear, but the fact that strong elevation of PI3-kinase does not activate ERK in COS cells indicates that PI3-kinase does not have an upstream regulatory role in the Raf-MEK-ERK signaling pathway (45). PI3-kinase may have a major role as a key regulator of early-phase differentiation of keratinocytes, as shown by the fact that blockage of PI3-kinase triggered differentiation, whereas activation of PI3-kinase prevented it (37). Thus EGF- and BDNF-activated PI3-kinase may support the mitogenic program by preventing the differentiation of the cells.

Induction of proliferation by extracellular signaling molecules involves the activation of gene transcription. Here, we have analyzed the EGF-, BDNF-, or A-Raf-induced biosynthesis of the transcription factors Egr-1 and c-Jun. Similar to the transient or sustained activation of ERK, we observed transient stimulation of Egr-1 biosynthesis by EGF or BDNF in HaCaTpac or HaCaT-TrkB cells, whereas a sustained synthesis of the transcription factor Egr-1 was detected in 4-OHT-treated HaCaT-D Aurora-F/ER cells. Nevertheless, the biological consequences of transient vs. sustained synthesis of Egr-1 were identical: the proliferation of HaCaT cells. Since the discovery of the Egr-1 gene as an early growth response gene, research has been directed toward elucidating the function of Egr-1 in growth and proliferation. Induction of Egr-1 gene transcription was monitored in many cell types in response to mitogens (42), and a direct role of Egr-1 during multistage carcinogenesis in the skin has been proposed (33). The fact that genes encoding growth factors, such as insulin-like growth factor II, platelet-derived growth factors A and B, and TGF-β1, have been identified as target genes of Egr-1 (24, 25, 40) indicates that Egr-1 may prolong the mitogenic signaling cascade by stimulation of growth factor synthesis. The proposed role for Egr-1 in controlling cell growth is, however, largely based on the correlation between mitogenic response and Egr-1 biosynthesis. Gain-of-function and loss-of-function experiments are required to decipher the exact role of Egr-1 in keratinocyte growth control. Moreover, the identification of Egr-1 target genes in keratinocytes should provide clues about how Egr-1 is performing its biological function.

The basic region leucine zipper protein c-Jun, one of the proteins that constitute the activator protein AP-1 transcription factor complex, plays an essential role in many cell types in controlling cell growth, survival, or death (19). The biological activity of c-Jun is regulated on several levels, including transcription of the c-Jun gene, the turnover rate of the mRNA and protein, and posttranslational modifications, as well as the interaction and dimerization with other basic region leucine zipper proteins. Here, we have shown that the c-Jun concentration is increased as a result of BDNF or EGF stimulation or activation of the A-Raf protein kinase. The growth factor-mediated increase in the c-Jun concentration is more moderate than the striking enhancement of Egr-1. However, the elevated c-Jun concentration is persistent compared with the short and transient synthesis of Egr-1. Gain-of-function and loss-of-function experiments may clarify whether BDNF, EGF, or A-Raf requires c-Jun as a positive regulator of proliferation in HaCaT cells.

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REFERENCES

PROLIFERATION OF HaCaT KERATOCYTES


