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Epidermal Growth Factor and Thrombin Induced Proliferation of Immortalized Human Keratinocytes is Coupled to the Synthesis of Egr-1, A Zinc Finger Transcriptional Regulator

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Abstract The epidermal growth factor (EGF) receptor is highly expressed in HaCaT keratinocytes as shown by Western blotting. Stimulation of HaCaT cells with EGF, and also with the serine protease thrombin, induced DNA synthesis, measured by incorporation of 5-bromo-2′-deoxyuridine into the DNA of proliferating cells. Using antibodies directed against the active form of the EGF receptor, we show that in HaCaT cells EGF and thrombin triggered a rapid activation of the EGF receptor, followed by the phosphorylation and activation of the extracellular signal-regulated protein kinase (ERK). Moreover, EGF and thrombin induced a transient synthesis of the zinc finger transcriptional regulator Egr-1. Proliferation, activation of ERK, and biosynthesis of Egr-1 was completely inhibited in EGF or thrombin-treated HaCaT cells by the MAP kinase kinase inhibitor PD98059 and by AG1487, an EGF receptor-specific tyrosine kinase inhibitor. These data indicate that phosphorylation and activation of both the EGF receptor and ERK are essential for mitogenic signaling via EGF and thrombin. The synthesis of Egr-1 in HaCaT cells as a result of EGF or thrombin stimulation suggests that Egr-1 may be an important ''late'' part of the EGF and thrombin-initiated signaling cascades. We postulate that Egr-1 may function as a ''third messenger'' in keratinocytes connecting mitogenic stimulation with changes in gene transcription. J. Cell. Biochem. 85: 381–391, 2002.

Key words: EGF; EGF receptor; Egr-1; HaCaT; extracellular signal-regulated kinase; signal transduction; thrombin

The epidermal growth factor (EGF) receptor belongs to the superfamily of receptors with intrinsic tyrosine kinase activity. Following binding of its cognate ligands, including EGF and transforming growth factor-α, the intrinsic kinase is activated and the receptor is tyrosyl-phosphorylated. The phosphotyrosyl residues are binding sites for adaptor proteins that interface with numerous downstream signaling pathways, including the activation of the mitogen-activated protein (MAP) kinase pathway via Ras, the activation of signal transducers and activators of transcription (STAT), and the activation of phospholipase Cγ. EGF receptor signaling has been intensely investigated in the last decade, making the EGF receptor a paradigm for receptor tyrosine kinases in general [Hackel et al., 1999; Moghal and Sternberg, 1999; Wells, 1999].

In epithelial cells, important roles of EGF receptor activation in controlling proliferation, differentiation, migration, and cell survival have been proposed [Jost et al., 2000]. In particular, the progression of human keratinocytes from the G1 to the S-phase of the cell cycle has been shown to be dependent on endogenous EGF-family growth factors [Kobayashi et al., 1998]. Likewise, inhibition of the EGF signaling pathway in keratinocytes reduced the survival of the cells [Stoll et al., 1998; Jost et al., 1999], suggesting that the EGF receptor-dependent

Abbreviations used: BrdU, 5-bromo-2′-deoxyuridine; EGF, epidermal growth factor; Egr-1, early growth response 1.

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signaling pathway is of major importance in the control of survial, cell proliferation, or cell death of keratinocytes.

The serine protease thrombin (EC 3.4.21.5) is the key enzyme in haemostasis. Thrombin catalyzes fibrin clot formation by converting fibrinogen into fibrin. Moreover, platelets are stimulated by thrombin to aggregation and secretion. In addition, thrombin functions as a mitogen for several cell types, including smooth muscle cells, fibroblasts, and keratinocytes [Shapiro et al., 1996; Balmanno and Cook, 1999; Algermissen et al., 2000]. Thrombin binds to seven-transmembrane-spanning G-protein coupled receptors termed protease activated receptors (PARs). These receptors are activated via proteolysis. The 41 N-terminal amino acids from the extracellular part of the receptor are cleaved off, generating a new N-terminus of the receptor protein that functions as a "tethered ligand" capable of activating its receptor [Grand et al., 1996; Coughlin, 1998, Goldsack et al., 1998]. HaCaT cells have been shown to express thrombin receptors. Moreover, a strong induction of proliferation was measured in thrombin-treated HaCaT keratinocytes [Algermissen et al., 2000].

We have studied EGF and thrombin signaling in HaCaT keratinocytes. The results show that both EGF and thrombin stimulation of HaCaT cells rapidly induce the biosynthesis of Egr-1, a transcriptional activator, suggesting that Egr-1 plays an integral part in EGF and thrombin induced proliferative signaling. Moreover, we show that the signaling cascades initiated by both EGF or thrombin includes the activation of the EGF receptor, followed by the activation of the ERK.

MATERIALS AND METHODS

Cell Culture

The human immortalized cell line HaCaT was a kind gift of N.E. Fusenig, Deutsches Krebsforschungszentrum, Heidelberg, Germany. The human brain glioma cell lines 1321N1 and U-373MG were obtained from the European Collection of Cell Cultures (ECACC # 86030402 and 89081402). The malignant astrocytoma cell line U343MG-a [Dirks et al., 1997] was a generous gift from J.T. Rudka, Division of Neurosurgery, Brain Tumor Research Laboratory, Hospital for Sick Children, Toronto, Canada. The human neuroblastoma cell lines SH-SY5Y, GOTO, and SMS-KCN cells were gifts of J. Biedler, Sloan–Kettering Institute for Cancer Research, New York, Emil Bogenmann, Childrens Hospital, Los Angeles, and G. M. Brodeur, Childrens Hospital of Philadelphia, respectively. Cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM glutamine (standard medium) at 37°C in 5% CO_2. Cells were stimulated with 10 ng/ml EGF (Promega, Mannheim, Germany, # G5021, dissolved in H_2O as a 100 μg/ml stock solution), 2 U/ml thrombin (Sigma, Steinheim, Germany, # T-6884, dissolved in DMEM at a concentration of 100 U/ml), or 1 μM vasoactive intestinal polypeptide (Sigma, #V-6130, dissolved in water). The MAP kinase kinase inhibitor PD98059 was purchased by Calbiochem (Darmstadt, Germany, # S513000), dissolved in DMSO, and used at a concentration of 50 μM. The EGF receptor-specific tyrosine kinase inhibitor AG1478 was also purchased by Calbiochem (# 658552), dissolved in DMSO, and used at a concentration of 0.5 μM. Cells were preincubated with AG1478 or PD98059 for 1 or 6 h, respectively.

Proliferation Assay

Cells were seeded in 96-well plates at a cell density of 2×10^4 cells per well in standard medium and incubated for 24 h. The serum concentration was lowered to 0.05% and the cells were incubated for another 24 h. Stimulation of the cells with EGF and thrombin was performed for 24 h. Induction of DNA synthesis was measured by the incorporation of the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells using the Cell Proliferation ELISA kit from Roche Diagnostics (Mannheim, Germany, # 1647229). 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 for 1 h 30 min. Peroxidase activity was determined spectrophotometrically as described in the instruction manual. Each experiment was performed in quintuplicates and the mean ± SEM is depicted.

Preparation of Cell Extracts

To prepare crude membrane, cells grown to 80–90% confluency in standard medium

were washed with ice cold PBS, harvested by scraping, and centrifuged at 200*g* for 10 min. The pellet was washed twice with PBS and lysis of the cells was performed in 2 ml of 20 mM HEPES, pH 7.4, containing 250 mM sucrose, 1 mM ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), and 470 μM phenylmethylsulfonyl-fluoride (PMSF) via sonication. Lysates were centrifuged at 4°C for 10 min at 1,250*g*. The supernatants were centrifuged again at 4°C for 1 h at 125,000*g*. The pellets, containing the crude membrane fraction, were finally dissolved in 25 mM HEPES, pH 7.4, containing 1% SDS. Protein was determined using the BCA kit from Pierce (# 23255). To detect the activated EGF receptor, HaCaT cells were first seeded at a density of 10^6 cells per 100 mm plate and incubated for 24 h. The medium was changed, the serum concentration reduced to 0.05%, and the cells were incubated for a further 24 h. Stimulation with 10 ng/ml EGF or 2 U/ml of thrombin was performed for 10 min. Crude membranes were prepared as described with minor modifications. The lysis buffer contained additionally 2 mM Na_3VO_4 and 10 mM NaF. Nuclear extracts were prepared from cells seeded in 100 mm plates at a density of 10^6 cells per plate in standard medium and incubated over night. The serum concentration was reduced to 0.05% and the cells were incubated for 24 h. Stimulation with 10 ng/ml EGF or 2 U/ml of thrombin was performed for 1, 3, 5, and 8 h. The cells were washed once with PBS and harvested. The cells were centrifuged at 15,000*g* for 10 min and the pellet was redissolved in 400 μl buffer containing 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM 1,4-dithio-DL-threitol (DTT), and 0.2 mM PMSF and incubated on ice for 10 min. The cells were vortexed, incubated for another 10 min and centrifuged for 1 min at 15,000*g*. The pellet was resuspended in 50 μl 20 mM HEPES-KOH, pH 7.9, containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF, incubated for 20 min on ice, and centrifuged for another 2 min to extract the nuclear proteins. The protein concentrations were determined using the BCA protein assay kit from Pierce. Whole cell extracts of EGF and thrombin stimulated cells were prepared by resuspending the washed cell pellets in 50 mM Tris, pH 8.0, containing 50 mM DTT, 1 mM PMSF, 2 mM Na_3VO_4, 10 mM NaF, and 1% SDS.

Antibodies and Immunoblot Analysis

About 10 μg of proteins from the crude membrane preparations were separated by SDS–PAGE on a 7.5% gel. The proteins were transferred to nitrocellulose membranes (Roche Diagnostics; Schleicher and Schuell, Dassel, Germany). Blots were incubated with antibodies directed against the EGF receptor (Santa Cruz, Heidelberg, Germany, # sc-03) or the 'active' EGF receptor (# E12120, Transduction Laboratories, Heidelberg, Germany). To detect the ERK, proteins derived from whole cell extract preparations were separated on SDS–PAGE and transferred to nitrocellulose membranes. Blots were probed with an antibody directed against the ERK (Santa Cruz, # sc-153) or the phosphorylated form of the kinase (Promega, # V8031). To analyze Egr-1 synthesis in EGF- or thrombin-stimulated cells, 10 μg of nuclear proteins were separated by SDS–PAGE on a 10% gel and the blots were incubated with an antibody directed against human Egr-1 (Santa Cruz, #sc-110). Blots were developed using a horseradish peroxidase conjugated goat anti-rabbit or anti-mouse secondary antibodies and ECL (Amersham, Freiburg, Germany).

RESULTS

EGF and Thrombin Induce Proliferation in HaCaT Cells

EGF functions as a powerful mitogen in many cell types of endodermal, mesodermal, and ectodermal origin. However, in A431 cells, which are derived from epidermoid carcinomas, EGF promotes cell cycle arrest [Gill and Lazar, 1981]. We therefore tested the effect of EGF upon proliferation of HaCaT cells. HaCaT cells are spontaneously-immortalized non-tumorigenic keratinocytes derived from normal-appearing black skin of a 62-year-old male [Boukamp et al., 1988]. As a molecular parameter for proliferation, we measured DNA synthesis via the incorporation of the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. The 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. The incorporation of BrdU into the DNA was detected by immunoassay. The cells were starved for 24 h and then treated with EGF for 24 h. Figure 1A shows that EGF induced a

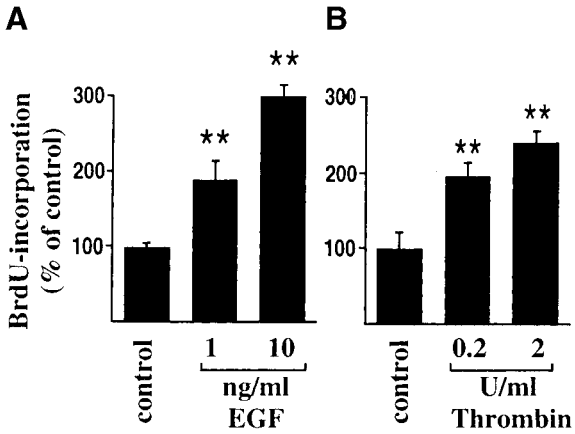

Fig. 1. Induction of DNA synthesis in HaCaT cells by EGF and thrombin. HaCaT cells were seeded in 96-well plates at a cell density of 2×10^4 cells per well in DMEM containing 10% fetal calf serum and incubated over night. The serum concentration was lowered to 0.05% and the cells were incubated for another 24 h. Stimulation of the cells with EGF (**A**) or thrombin (**B**) was performed for 24 h using the concentrations indicated. Induction of DNA synthesis was measured by the incorporation of the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU), instead of thymidine, into the DNA of proliferating cells. The cells were incubated with BrdU for 2 h and the incorporation of BrdU into the DNA was detected by immunoassay. At least two experiments were performed in quintuplicates and the mean ± SEM is depicted. **, values statistically significant different from controls at $P < 0.005$.

significant increase in BrdU incorporation in HaCaT cells, indicating that EGF functions as a mitogen for these cells. Stimulation of HaCaT cells with thrombin had been shown to induce tyrosine phosphorylation of the EGF receptor [Daub et al., 1997], suggesting that the EGF receptor represents an essential link in G-protein coupled thrombin-receptor induced signal transduction. We tested the mitogenic activity of thrombin for HaCaT cells. Figure 1B shows that thrombin efficiently induced DNA synthesis. We conclude that both EGF and thrombin function as mitogens for HaCaT cells. In contrast to previous published reports [Sung et al., 1999; Granoth et al., 2000], we did not observe an effect of vasoactive intestinal polypeptide on HaCaT cell proliferation (data not shown).

EGF Receptor Expression Level in HaCaT Cells

We performed Western blot experiments with crude membranes prepared from HaCaT cells to estimate the EGF receptor expression level in HaCaT cells. For comparison, we prepared membranes from the human glioma cell lines 1321N1, U343MG-a, and U-373MG and the human neuroblastoma cell lines SH-SY5Y, GOTO, and SMS-KCN. Figure 2A shows that the EGF receptor is strongly expressed in HaCaT cells in comparison to the moderate expression levels found in human neuroblastoma cells. The expression level of the EGF receptor in HaCaT cells was even comparable to the one found in some human glioma cell lines known to express high levels of the receptor [Libermann et al., 1985; Wong et al., 1987, Kaufmann and Thiel, 2001].

Detection of the Activated Form of the EGF Receptor in HaCaT Cells Following Stimulation With EGF or Thrombin

Following binding of the ligand, EGF receptors oligomerize and activate their intrinsic tyrosine kinase activity, leading to autophosphorylation. Activated EGF receptors are therefore frequently detected with anti-

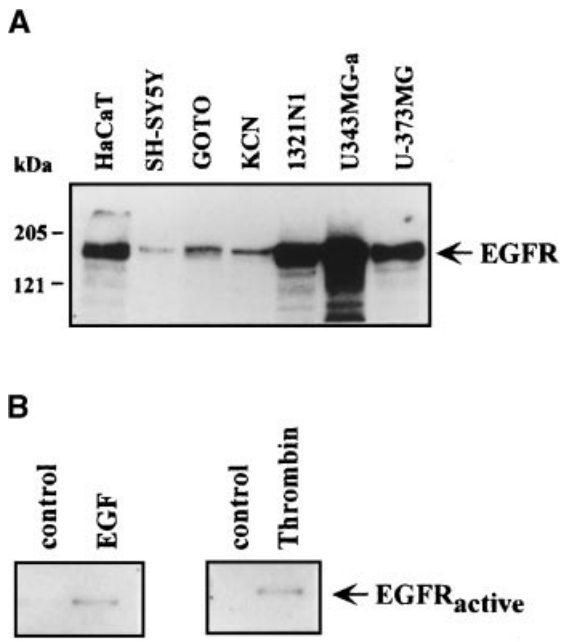

Fig. 2. EGF receptor expression levels in human HaCaT. **A**: Crude membranes were prepared from HaCaT cells. For comparison, membranes from the human neuroblastoma cell lines SH-SY5Y, GOTO, and SMS-KCN, and the human glioma cell lines 1321N1, U343MG-a, and U-373MG were also analyzed. 10 μg of crude membrane proteins were separated on a 7.5% SDS–PAGE gel and blotted to a nitrocellulose membrane. The membranes were probed with anti-serum directed against the EGF receptor. **B**: HaCaT cells were serum-starved for 24 h, treated either with 10 ng/ml EGF or 2 U/ml of thrombin for 10 min or left untreated (control). Crude membranes were prepared and subjected to Western blot analysis. The blot was incubated with a monoclonal antibody against the activated EGF receptor.

phosphotyrosine antibodies. Here, we used an antibody that specifically recognized the conformation of the activated EGF receptor [Campos-Gonzales and Glenney, 1991; Emlet et al., 1997]. The results show that EGF treatment of HaCaT cells induced the active conformation of the EGF receptor (Fig. 2B). Likewise, thrombin was able to activate the EGF receptor in HaCaT cells, confirming earlier reports performed with phosphotyrosine-specific antibodies that described the transactivation of the EGF receptor by thrombin [Daub et al., 1997].

Phosphorylation and Activation of MAP Kinase Following EGF or Thrombin Treatment of HaCaT Cells

The activation of the EGF receptor signaling cascade is known to trigger the phosphorylation and activation of ERKs, ERK1, and ERK2 [Walker et al., 1998]. We used an antibody directed against the phosphorylated form of ERK2 to detect signal-induced activation of ERK. The antibody mainly detected the activated p42 isoform of ERK termed ERK2. As shown in Figure 3, administration of EGF or thrombin triggered a rapid phosphorylation, i.e., activation of ERK2 in HaCaT cells. EGF or thrombin treatment did not affect the level of total ERK expression in HaCaT cells as shown in Figure 3 (lower panel), as detected with an antibody that was not specific for the phosphorylation-status of the protein kinase.

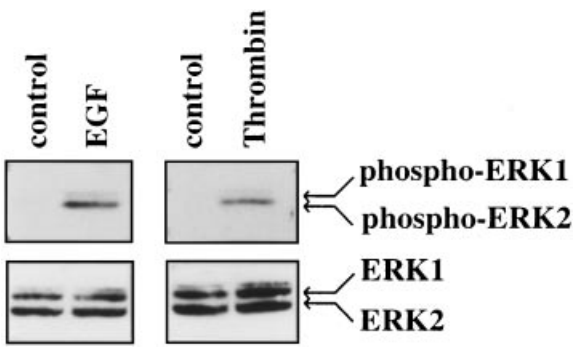

Fig. 3. Phosphorylation and activation of ERK in HaCaT cells following stimulation with EGF or thrombin. HaCaT cells were serum-starved for 24 h, treated with 10 ng/ml EGF or 2 U/ml of thrombin for 10 min or left untreated (control). Whole cell extracts were prepared and subjected to Western blot analysis. The blot was incubated with an affinity purified rabbit antibody directed against the phosphorylated active forms pp42 (ERK2) (upper panels) or an antibody that detects ERK independently of the phosphorylation status of the protein (lower panels).

EGF and Thrombin Stimulate the Biosynthesis of Egr-1 in HaCaT Cells

The mitogenic effects of EGF and thrombin upon HaCaT cells ultimately requires changes of the gene expression pattern of the cells. To follow the EGF and thrombin signaling cascade, we analyzed the expression of the zinc finger transcription factor Egr-1. Transcription of the Egr-1 gene has been shown to be induced by mitogens in various cell types [Gashler and Sukhatme, 1995]. To test the effects of EGF and thrombin on Egr-1 biosynthesis, HaCaT cells were serum-starved for 24 h and then incubated with 10 ng/ml EGF or 2 U/ml thrombin for 1, 3, 5, and 8 h. The cells were harvested and nuclear extracts prepared. Egr-1 expression of HaCaT cells treated with EGF or thrombin was analyzed with antibodies directed against Egr-1. Weak Egr-1 immunoreactivity was observed in the absence of the stimuli. In contrast, EGF (Fig. 4A) and thrombin (Fig. 4B) strikingly increased the biosynthesis of Egr-1 with a peak of expression between 1 and 5 h following stimulation with EGF. Highest amounts of Egr-1 were observed after 1 h of stimulation with thrombin, indicating that EGF and thrombin slightly differ in their abilities to induce Egr-1-

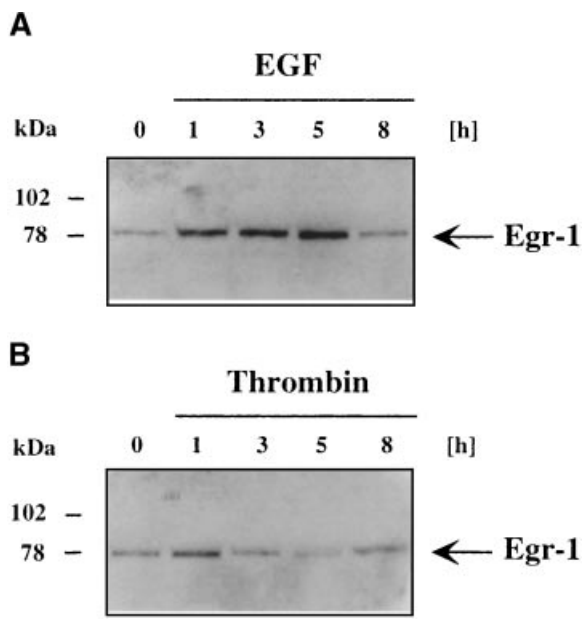

Fig. 4. EGF and thrombin stimulate the biosynthesis of Egr-1 in human HaCaT cells. HaCaT cells were stimulated with 10 ng/ml of EGF (**A**) or 2 U/ml of thrombin (**B**). Nuclear extracts were prepared from different time points and the proteins analyzed by immunoblotting for Egr-1 immunoreactivity.

biosynthesis. While the induction of Egr-1 synthesis by thrombin was transient, we observed increased Egr-1 immunoreactivity for 5 h following stimulation of the cells with EGF. The transient induction of Egr-1 by thrombin was not prolonged by using higher thrombin concentrations (data not shown).

Role of EGF Receptor Activation in Thrombin-Induced ERK Activation, Egr-1 Synthesis, and Cell Proliferation

Thrombin has been shown to change the conformation of the EGF receptor to its activated state. To verify that activation of the EGF receptor is crucial for the thrombin-induced activation of ERK and the subsequent biosynthesis of Egr-1, we treated HaCaT cells with the tyrosine kinase inhibitor AG1478 before we stimulated the cells with thrombin. The results show that the thrombin-induced activation of ERK2 (Fig. 5A), the synthesis of Egr-1 (Fig. 5B) as well as the stimulation of DNA synthesis (Fig. 5C) is completely blocked in cells preincubated with this compound, indicating that the EGF receptor is a necessary link for thrombin-mediated signaling in HaCaT cells. As a control, we tested the effects of AG1478 upon EGF signaling in HaCaT cells. Naturally, AG1478 totally blocked EGF signaling in HaCaT cells.

EGF and Thrombin Induced Signaling in HaCaT Cells Requires the Activation of ERK

The activated EGF receptor is not exclusively coupled to the Ras/Raf/ERK signaling pathway. Additionally, an EGF receptor-dependent activation of the STAT family of gene regulatory proteins has been described [Fu and Zhang, 1993; Ruff-Jamison et al., 1994]. The EGF

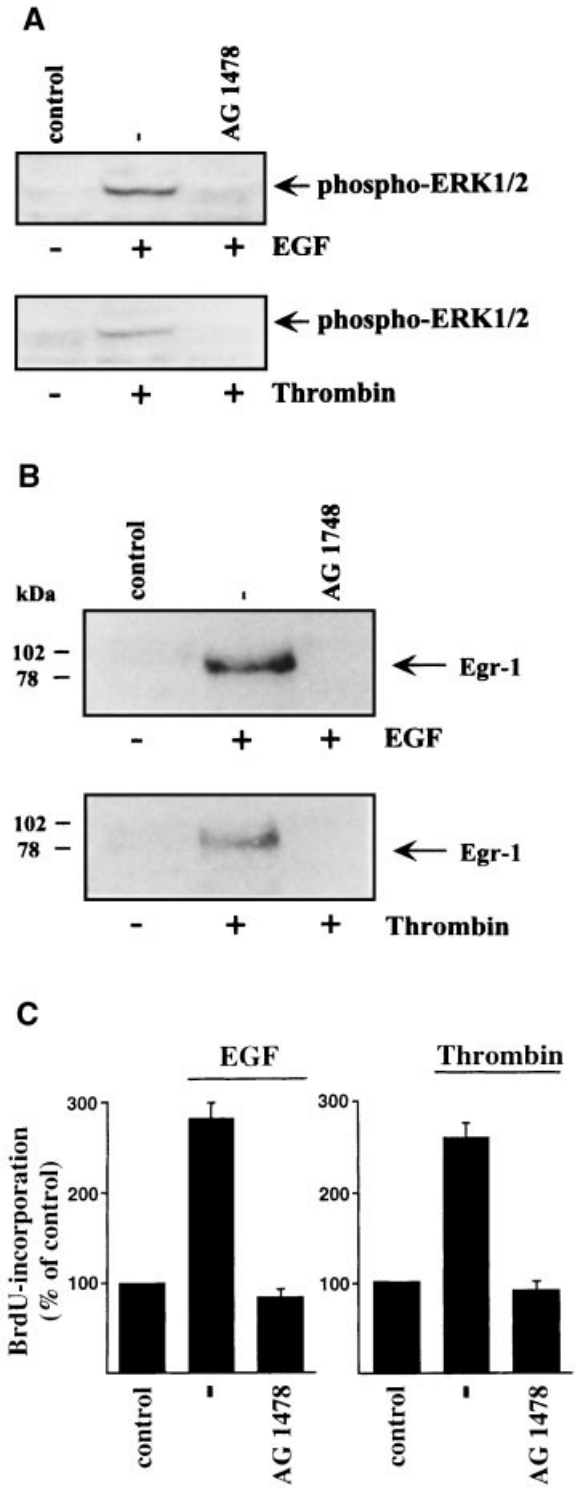

Fig. 5. Role of EGF receptor activation on thrombin-induced signal transduction in HaCaT keratinocytes. HaCaT cells were preincubated for 1 h with the tyrophostin AG1487 and then stimulated with 10 ng/ml of EGF (upper panel), 2 U/ml of thrombin (lower panel), or vehicle (control). Whole cell extracts or nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against the phosphorylated active form pp42 (ERK2) (**A**) or against Egr-1 (**B**). **C**: HaCaT cells were seeded in 96-well plates at a cell density of 2×10^4 cells per well in DMEM containing 10% fetal calf serum and incubated over night. The serum concentration was lowered to 0.05%, and the cells were incubated for another 24 h. Cells were preincubated with AG1487 for 1 h, then stimulated with EGF or thrombin for 24 h. Induction of DNA synthesis was measured as described in the legend to Figure 1.

receptor dependent tyrosine phosphorylation and nuclear translocation of STAT1 activates the c-fos gene promoter. Both Egr-1 and c-fos genes are immediate-early response genes that show many similarities in their regulation. To verify the role of ERK in EGF and thrombin induced Egr-1 biosynthesis and cell proliferation and to exclude signaling via STATs, we preincubated the cells with PD98059. This compound inhibits phosphorylation of the MAP kinase kinase, thus blocking the activation of ERK [Dudley et al., 1995]. As expected, PD98059 blocked phosphorylation and activation of ERK in EGF and thrombin treated HaCaT cells (Fig. 6A). Moreover, preincubation of HaCaT cells with PD98059 impaired EGF or thrombin-stimulated synthesis of Egr-1 (Fig. 6B), indicating that the activation of ERK is required for Egr-1 gene transcription. Finally, we studied the effects of PD98059 on HaCaT cell proliferation. The cells were preincubated with PD98059 and then stimulated with EGF or thrombin. DNA synthesis was measured via incorporation of BrdU into DNA. Fig. 6C shows that PD98059 efficiently blocked the BrdU incorporation into DNA of HaCaT cells that had been stimulated with EGF or thrombin, indicating that the mitogenic activity of EGF and thrombin is mediated by an activated ERK.

DISCUSSION

We have shown that the EGF receptor is highly expressed in the immortalized keratinocyte cell line HaCaT and that receptor stimulation induces cell proliferation. The fact that EGF receptor expression and activation is deregulated in benign and malignant hyperproliferative skin diseases underlines the

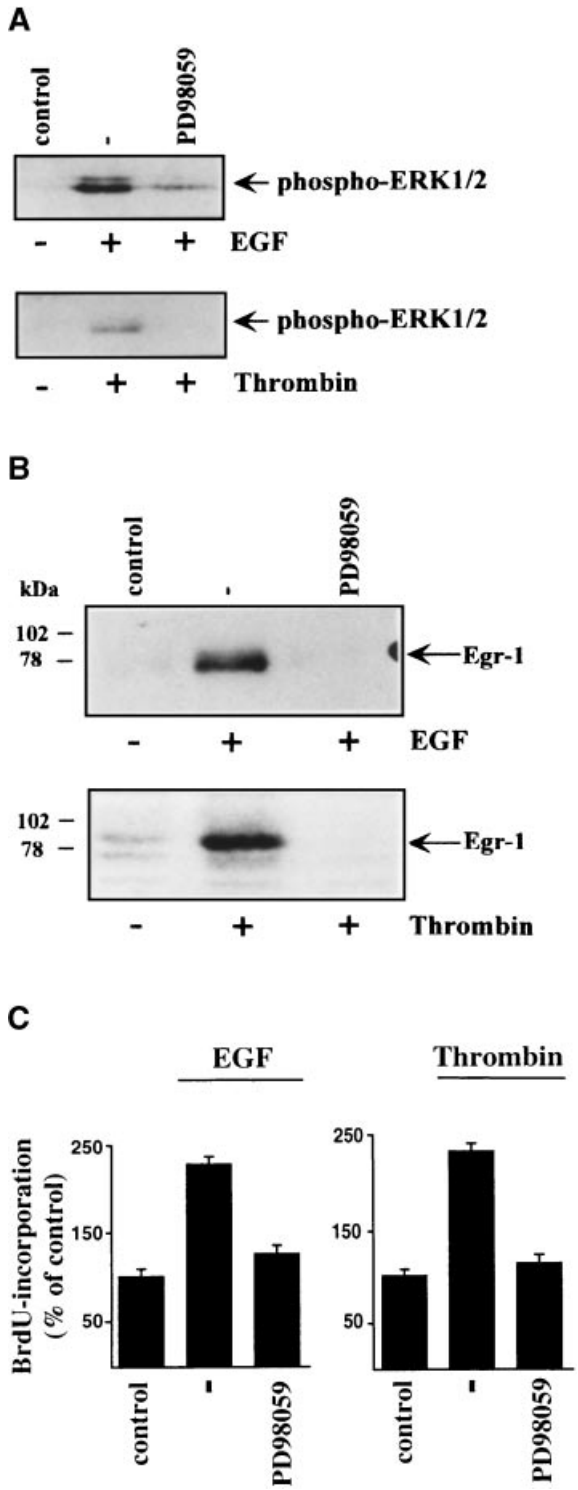

Fig. 6. Effects of PD98059 on EGF or thrombin induced signal transduction in HaCaT keratinocytes. HaCaT cells were preincubated for 6 h with the MAP kinase kinase inhibitor PD98059 and then stimulated with 10 ng/ml of EGF (upper panel), 2 U/ml of thrombin (lower panel), or vehicle (control). Whole cell extracts and nuclear extracts were prepared and subjected to Western blot analysis using either an antibody directed against the phosphorylated active form pp42 (ERK2) (**A**) or against Egr-1 (**B**). **C**: HaCaT cells were seeded in 96-well plates at a cell density of 2×10^4 cells per well in DMEM containing 10% fetal calf serum and incubated over night. The serum concentration was lowered to 0.05% and the cells were incubated for another 24 h. Cells were preincubated with PD98059 for 6 h, then stimulated with EGF or thrombin for 24 h. Induction of DNA synthesis was measured as described in the legend to Figure 1.

importance of EGF signaling for keratinocyte survival. Inhibition of EGF receptor activation has been shown to induce extensive cell death in keratinocyte cultures [Rodeck et al., 1997]. Vice versa, EGF signaling inhibits programmed cell death of keratinocytes [Stoll et al., 1998; Gibson et al., 1999; Jost et al., 2001], indicating that EGF receptor stimulation of epithelial cells has a significant survival function. These data indicate that the EGF receptor-dependent signaling pathway is of major importance in the control of survival, cell proliferation, or cell death of keratinocytes. Transgenic mice lacking the EGF receptor are growth retarded and die during development. The mutant mice show defects in epithelial development, further indicating that the EGF receptor is essential in epithelial cell proliferation and differentiation [Miettinen et al., 1995; Sibilia and Wagner, 1995].

The mitogenic signaling cascade initiated by thrombin was shown here to involve the activation of the EGF receptor, the phosphorylation and activation of ERK, and, most likely, the biosynthesis of the transcriptional regulator Egr-1. In comparison to the EGF-triggered signaling cascade, we observed a transient activation of the Egr-1 biosynthesis with highest amounts of Egr-1 synthesized 1 h after stimulation thrombin. The biological outcome of transient versus sustained activation of Egr-1 biosynthesis is currently not known. Experiments are in progress using an inducible Egr-1 mutant to clarify this issue. The activation of the G-protein coupled PARs by thrombin activates the tyrosyl-phosphorylation of the EGF receptor in various cell types, including HaCaT cells [Daub et al., 1997]. Here, using a monoclonal antibody that recognized the activated conformation and not the phosphotyrosine content of the EGF receptor, we demonstrate that thrombin-stimulation of its cognate G-protein coupled receptor is followed by an activation of the EGF receptor. The conformational change induced by thrombin was indistinguishable from that induced by EGF that targets the EGF receptor directly. The EGF receptor is a substrate for cytosolic tyrosine kinases of the src-family and it has been suggested that the activation of PARs activate those kinases that subsequently phosphorylate the EGF receptor [Leserer et al., 2000].

The signaling cascades initiated by EGF and thrombin in HaCaT cells were further analyzed. The activation of the EGF receptor is frequently coupled to the activation of the Ras/ERK signaling pathway, leading finally to the phosphorylation and activation of ERK. Using phosphospecific antibodies, we showed that EGF and thrombin induced phosphorylation of ERK in HaCaT cells that was impaired by PD98059, a MAP kinase kinase inhibitor. An impairment of ERK phosphorylation and activation by PD98059 has also been demonstrated in EGF-stimulated T47D breast adenocarcinoma cells [Gibson et al., 1999]. The fact that EGF and thrombin-stimulated cell proliferation was blocked by PD98059 indicates that the activation of ERK is absolutely essential for the mitogenic activities of EGF and thrombin. Thus, the intrinsic EGF receptor kinase activity is necessary to transmit signals from activated G-protein coupled receptors to the Ras/ERK signaling pathway. Similar observations have been reported for lung fibroblasts and airway smooth muscle cells [Shapiro et al., 1996; Trejo et al., 1996]. The activated EGF receptor is, however, not exclusivley coupled to the Ras/ERK signaling pathway. The EGF receptor can directly activate a pool of latent cytoplasmic transcription factors termed STATs. In some cell types, STAT proteins are phosphorylated on tyrosine residues as a result of EGF receptor activation. The proteins subsequently translocate into the nucleus to activate gene transcription of their cognate target genes. In A431 squamous carcinoma cells, EGF was shown to induce the expression of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 via the STAT pathway, leading to growth inhibition [Chin et al., 1996]. In these cells, therefore, the STATs play a negative role in the control of cell proliferation, in contrast to the positive role executed by the mitogen-induced Ras/ERK pathway. In HaCaT cells, ERK activation is essential for proliferation, indicating that the activated EGF receptor couples with the Ras/ERK signaling pathway. Stimulation of HaCaT cells with EGF was also shown to have no impact upon STAT3 phosphorylation [Jost et al., 2001]. In contrast, in transformed sqamous epithelial cells EGF stimulation induces the phosphorylation of STAT3 [Grandis et al., 1998]. Likewise, in HaCaT cells EGF did not induce phospholipase Cγ phosphorylation or the activation of the protein kinase AKT [Jost et al., 2001], indicating that EGF receptor activation in HaCaT cells is coupled to the Ras/ERK signaling pathway, and not to signaling

cascades involving AKT, phospholipase Cγ, or STATs.

Induction of cell proliferation requires an alteration in the genetic program of HaCaT cells. One of the targets of the ERK signaling pathway is the gene encoding the transcriptional regulator Egr-1. A variety of environmental signals, including growth factors, induce Egr-1 gene expression [Gashler and Sukhatme, 1995; Khachigian and Collins, 1998] suggesting that Egr-1 couples extracellular signals to long-term responses by altering expression of Egr-1 target genes. Since the discovery of the Egr-1 gene as an "early growth response gene" [Sukhatme et al., 1988], research was directed towards a function of Egr-1 in growth and proliferation. In fact, induction of Egr-1 gene transcription was monitored in many cell types in response to mitogens [Gashler and Sukhatme, 1995] and a direct role of Egr-1 in controlling proliferation has been proposed for T-cells, astrocytes, glioma cells, and glomerular mesangial cells [Perez-Castillo et al., 1993; Biesiada et al., 1996; Hofer et al., 1996; Kaufmann and Thiel, 2001]. Recently, the analysis of the effect of Egr-1 deficiency on tumor development in two transgenic mouse models of prostate cancer revealed that tumor progression was significantly impaired in Egr1−/− mice [Abdulkadir et al., 2001]. Furthermore, a role of Egr-1 during multistage carcinogenesis in the skin has been proposed [Riggs et al., 2000]. Mitogens induce a transient synthesis of Egr-1, as shown here for EGF and thrombin. Egr-1 functions as a transcription factor that activates downstream target genes that are responsible for the alterations of the gene expression pattern required for proliferation. The identification of those downstream target genes of Egr-1 will help us in the understanding of the entire signal-transcription coupling mediated by Egr-1. Recently, a search for Egr-1 target genes in prostate carcinoma cells using the microarray technology identified several genes encoding growth factors such as insulin-like growth factor-II, platelet-derived growth factor-A, and transforming growth factor-β1 [Svaren et al., 2000], suggesting that Egr-1 may continue the mitogenic signaling cascade via the stimulation of growth factor synthesis. The identification of Egr-1 target genes in keratinocytes will be of interest to elucidate the EGF and thrombin induced signaling cascade in this cell type downstream of Egr-1. In addition, an investigation of the kinetics of transcriptional activation of Egr-1-responsive genes should elucidate whether a transient synthesis of Egr-1 is sufficient to continue the signaling cascade.

The Egr-1 5′-flanking region contains five serum response elements (SRE) that function as binding sites for the serum response factor SRF and for a factor that forms a ternary complex with the SRF protein. The ternary complex factor Elk1 is phosphorylated by ERK, connecting the activated ERK signaling pathway with enhanced transcription of genes containing SREs in their regulatory region. For the Egr-1 promoter, we showed that expression of a constitutively active mitogen-activated kinase kinase-1, the kinase responsible for the phosphorylation and activation of ERK, strongly stimulated the activity of the Egr-1 promoter, explaining the effects of mitogens upon Egr-1 transcription [Kaufmann et al., 2001].

The activation of ERK is accompanied by subsequent synthesis of the Egr-1 protein, as revealed by immunoblotting experiments. Thus, EGF receptor activation, by EGF or thrombin, in HaCaT cells is tightly connected with Egr-1 synthesis. Moreover, the activation of the ERK signaling pathway not only stimulates the biosynthesis of Egr-1, but also enhances the transcriptional activity of Egr-1 [Kaufmann et al., 2001]. Loss-of-function experiments of Egr-1 are necessary to uncover the role of Egr-1 in EGF and thrombin signaling and control of proliferation. To neutralize the biological activity of Egr-1, overexpression experiments of the negative cofactors, NAB1 or NAB2, may be useful. NAB1 and NAB2 are negative cofactors of Egr-1 that are able to completely block transcriptional activation mediated by Egr-1 [Russo et al., 1995; Svaren et al., 1996; Thiel et al., 2000]. The concentration of NAB1 and the related protein NAB2 in a particular cell is thus of extreme importance for Egr-1 function.

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