Cloning and Biological Activity of Epigen, a Novel Member of the Epidermal Growth Factor Superfamily

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High throughput sequencing of a mouse keratinocyte library was used to identify an expressed sequence tag with homology to the epidermal growth factor (EGF) family of growth factors. We have named the protein encoded by this expressed sequence tag Epigen, for epithelial mitogen. Epigen encodes a protein of 152 amino acids that contains features characteristic of the EGF superfamily. Two hydrophobic regions, corresponding to a putative signal sequence and transmembrane domain, flank a core of amino acids encompassing six cysteine residues and two putative N-linked glycosylation sites. Epigen shows 24–37% identity to members of the EGF superfamily including EGF, transforming growth factor α, and Epiregulin. Northern blotting of several adult mouse tissues indicated that Epigen was present in testis, heart, and liver. Recombinant Epigen was synthesized in Escherichia coli and refolded, and its biological activity was compared with that of EGF and transforming growth factor α in several assays. In epithelial cells, Epigen stimulated the phosphorylation of c-erbB-1 and mitogen-activated protein kinases and also activated a reporter gene containing enhancer sequences present in the c-fos promoter. Epigen also stimulated the proliferation of HaCaT cells, and this proliferation was blocked by an antibody to the extracellular domain of the receptor tyrosine kinase c-erbB-1. Thus, Epigen is the newest member of the EGF superfamily and, with its ability to promote the growth of epithelial cells, may constitute a novel molecular target for wound-healing therapy.

The epidermis of mammalian skin is a complex structure, the assembly and maintenance of which requires the regulation of a large number of genes (1). In particular, mRNAs encoding several members of the EGF superfamily of growth factors have been localized to the proliferative compartment of the epidermis, suggesting that they play an important role in the maintenance of skin structure (2–4). The EGF superfamily is an expanding group of growth factors containing several members including EGF, TGFα, Epiregulin, HB-EGF, AR, betacellulin, and the neuregulins (5–11). These members were first identified as secreted peptides; however, subsequent cloning of their cDNA has revealed that all are derived from membrane-bound precursors that are proteolytically cleaved from the plasma membrane (9, 12–16). Although members of the EGF superfamily have relatively low homology with each other at the amino acid level, the presence of six conserved cysteine residues in the active peptide suggests that all have a similar tertiary structure. Indeed, the solution structure of EGF and TGFα demonstrated that identical disulfide linkages between these conserved cysteine residues enable the formation of a three-looped structure (17). Central to the function of the EGF superfamily is a conserved domain known as the EGF motif, which is present in all EGF superfamily members identified to date. This motif encompasses three of the six conserved cysteine residues and contains additional residues important for tertiary structure stabilization and receptor binding (18).

Just as the EGF superfamily comprises structurally similar members, so do the receptors through which the peptides signal. In mammals, the EGF receptor tyrosine kinase family includes c-erbB-1 (EGFR, HER-1), c-erbB-2 (HER-2, Neu), c-erbB-3 (HER-3), and c-erbB-4 (HER-4) (19–22). c-erbB receptor signal transduction begins with stabilization of a receptor homo- or heterodimer through ligand binding (23). Signaling is such that a single receptor, e.g. c-erbB-1, can bind to several EGF superfamily ligands (EGF, TGFα, epiregulin, AR, HB-EGF, betacellulin; see Ref. 24 for review), and a single EGF superfamily ligand (e.g. epiregulin) can bind to several receptors (c-erbB-1 and B-4; Ref. 25). Tyrosine phosphorylation of C-terminal residues of c-erbB receptors activates the Ras-MAP kinase pathway, leading to regulation of c-fos expression by the binding of transcription factors to sites such as the serum inducing element and SRE within the c-fos promoter (see Ref. 26 for review).

The Ras-MAP kinase signaling pathway is widely used by receptor tyrosine kinases to promote diverse cellular responses including cell growth, differentiation, and apoptosis (27, 28). In vitro analysis of EGF superfamily members has revealed that all are able to stimulate or inhibit the proliferation of epithelia-derived normal and transformed cell lines, and several members are able to stimulate growth of fibroblasts, smooth muscle, and neural cell lines in culture (11, 29–35). EGF and TGFα can promote angiogenesis in vivo and can stimulate migration of endothelial cells and keratinocytes in vitro, both features thought to be of importance in epithelial wound healing (36–41). However, aberrant activation of the EGF ligand/receptor pathway in diseases such as psoriasis and cancer suggests that disregulation of EGF superfamily members and their receptors...
may play a role in the progression of these and other disorders (32, 42–46).

We have identified a novel member of the EGF ligand superfamily from mouse keratinocytes, which we have named Epigen, for epithelial mitogen. mRNA encoding Epigen has a restricted tissue distribution, present in heart, liver, and testis. We have purified recombinant Epigen and compared its biological activities with that of EGF and TGFα. In epithelial cells, Epigen stimulates the phosphorylation of c-erbB-1 and MAP kinase proteins. Epigen also activates genes under the control of the SRE. In addition, Epigen is a mitogen for HaCaT cells, and this activity can be significantly reduced by a blocking antibody to the receptor c-erbB-1.

**EXPERIMENTAL PROCEDURES**

*Cell Culture*—HaCaT and A431 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Life Technologies, Inc.), 0.77 mM L-asparagine (Sigma), 0.2 mM arginine (Sigma), 160 mM penicillin G (Sigma), 70 mM dihydrostreptomycin sulfate (Roche Molecular Biochemicals). HaCaT SRE cells were also supplemented with 0.5 mg/ml geneticsin (Life Technologies, Inc.).

*PCR Amplification*—A population of basal keratinocytes from the epidermis of neonatal mouse skin was used to generate a directionally cloned cDNA library in pBK-CMV using the Zap Express kit (Stratagene). Clones excised from the library were sequenced from the T3 primer, and homology searches were performed using the FastA and FastaX algorithms.

*Northern Blotting*—An adult mouse tissue Northern blot was purchased from CLONTECH and probed with a [32P]dATP end-labeled antisense oligonucleotide corresponding to the sequence 5′-GGTCGTGATATAGGACACCGACTCATTTCCTGCTCTGGATCTGTTG-3′ according to the manufacturer’s instructions. The blot was reprobed using the control β-actin fragment provided with the blot.

*Bacterial Construction for Protein Expression*—For bacterial expression, the vector pET16b (Novagen) was modified to shorten the bacterial leader sequence. The vector was digested with NcoI and XhoI to remove the existing leader sequence. This was replaced by the sequence 5′-ccatgggcattccatcaccaccatgcgaattcgctcgag-3′, which contains the eukaryotic poly(A) sequence MGGHHHHHANSLS. DNA encoding residues 53–103 of Epigen was polymerase chain reaction-amplified using the primers 5′-ggatccgtgaacctgctgacgcagatgt-3′ and 5′-gatctagggctacagggaaaggccg-3′, which match the consensus sequence established for eukaryotic ATG initiation codon (AATAAA). Conceptual translation of the 459-base frame of 459 nucleotides (Fig. 1A) was found upstream of the ATG codon in this cDNA, whereas a poly(A) stretch preceded by a putative polyadenylation signal (AATAAA).

**RESULTS**

*A Novel EGF Family Member Isolated from a Mouse Keratinocyte cDNA Library*—High throughput screening of a mouse immature keratinocyte library identified an expressed sequence tag of 1715 base pairs containing a single open reading frame of 459 nucleotides (Fig. 1A). A putative translation initiation codon was found within the sequence GAAATGGG, which matched the consensus sequence established for eukaryotic translational initiation (47). Only 5 base pairs of sequence were found upstream of the initiation codon. There is an 18-base untranslated region and the ATG is followed by a 25-base in-frame poly(A) stretch preceded by a putative polyadenylation signal (AATAAA).

**Phosphorylation Assays**—For MAP kinase phosphorylation assays, HaCaT cells were seeded in 6-well dishes such that the cells reached 80% confluence after an overnight incubation. Cells were then transferred into serum-free media and incubated for a further 24 h. Recombinant Epigen, TGFα (Genzyme), or control protein (TR1002P, a secreted protein produced as for Epigen) was added to the cells at a concentration of 18 nM and incubated for up to 20 min. Media was removed after the indicated times, and cells were immediately lysed in 100 μl of radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5 mM sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, pepstatin). MAP kinase phosphorylation was then assessed by SDS-PAGE and Western blot analysis using anti-activating MAP kinase polyclonal antibody (Promega). Western blots were stripped and reprobed using anti-ERK1/2 antibody (Santa Cruz Biotechnology) to ensure equal protein loading. For c-erbB-1 phosphorylation assays, A431 cells were substituted for HaCaT cells and treated in the same manner. c-erbB-1 phosphorylation was assessed as above, using an antibody to EGF receptor (activated form, Transduction Laboratories) following the manufacturer’s instructions. Blots were stripped and reprobed using anti-EGF receptor (Transduction Laboratories) to ensure equal protein loading.

*SRE Activation*—HaCaT cells were stably transfected with the cotransfected SRE construct described above, using standard techniques. For the assay, 5 × 104 cells were aliquoted into wells of a 96-well plate and incubated for 24 h. Media was changed to 0.1% fetal bovine serum containing cells for an additional 6 days in an atmosphere containing 10% CO2 at 37 °C. Cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction. HaCaT inhibition assays were performed essentially as above except that anti-c-erbB-1 (USB) or isotype control antibodies were titrated into wells (from 1 μg/ml) containing a constant amount of Epigen (1.75 nM) or TGFα (0.1 nM).

**RESULTS**

A Novel EGF Family Member Isolated from a Mouse Keratinocyte cDNA Library—High throughput screening of a mouse immature keratinocyte library identified an expressed sequence tag of 1715 base pairs containing a single open reading frame of 459 nucleotides (Fig. 1A). A putative translation initiation codon was found within the sequence GAAATGGG, which matched the consensus sequence established for eukaryotic translational initiation (47). Only 5 base pairs of sequence were found upstream of the initiation codon. There is an 18-base untranslated region and the ATG is followed by a 25-base in-frame poly(A) stretch preceded by a putative polyadenylation signal (AATAAA). Conceptual translation of the 459-base open reading frame encoded a predicted protein of 152 amino acids. Hydrophobicity analysis identified a stretch of 18 amino acids, starting from the putative initiating methionine, indicative of a signal peptide. The putative cleavage site of the
signal peptide was predicted to be located between Ala18 and Ala19 (SignalP; Ref. 48). An additional hydrophobic region was present between Ile111 and Cys130, suggesting the presence of a transmembrane domain. A search against the Prosite data base (release 15.0; Ref. 49) revealed that residues 83–94 (CRCFTGYTGQRC) matched the consensus pattern of EGF 1 and 2 domains. In addition, the predicted protein also contained two N-linked glycosylation sites between amino acids 36 and 39 (NWTF) and between amino acids 40 and 43 (NNTE).

Alignment of the predicted protein, which we have named Epigen, with entries in the Swiss-Prot Database indicated that Epigen was similar to several members of the EGF family. Over the entire Epigen protein sequence, this homology was relatively low, displaying 26 and 29% identity to TGFα and Epiregulin, respectively. This compares favorably with the protein sequence identity between other EGF family members, which ranges from 23 to 33%. Because EGF family members exist in a functional form as small peptides, we aligned the functional peptides of the EGF family with Epigen (Fig. 1B). This revealed that a 51-amino acid internal segment of Epigen was more than 40% identical to the active peptides of EGF, TGFα, and Epiregulin. The active peptides of the EGF family are sufficient for activity and contain several conserved residues critical for the maintenance of this activity. Epigen has also retained these residues, which include six cysteines (Cys59, Cys67, Cys72, Cys83, Cys85, Cys94), three glycines (Gly70, Gly88, Gly91), and an arginine and leucine (Arg93 and Leu99). The six cysteine residues in TGFα form three disulfide bonds that are required for optimum binding affinity to c-erbB-1 (50, 51).

Mutation of Arg93 and Leu99 was found to abrogate binding of EGF and TGFα to c-erbB-1 (18), and Gly91 and Arg93 are highly conserved within EGF family members but not in proteins that contain EGF units without growth factor activity (52). Together, the conserved features of Epigen with members of the EGF family at the amino acid level suggested that Epigen encoded a novel member of the EGF family. Furthermore, an increase in sequence conservation of a 51-amino acid internal
Epigen was cloned into a bacterial expression vector for protein production in whole bacterial lysates before (Fig. 3, A–C). A schematic representation of the complete coding region of Epigen (not drawn to scale). DNA encoding amino acids 53–103 of Epigen were subcloned into a bacterial expression vector for protein purification. The one-letter amino acid code has been used. B, recombinant Epigen production in whole bacterial lysates before (lane 1) and after (lane 2) induction by isopropyl-1-thio-β-D-galactopyranoside and detection by Western blotting using an anti-His antibody. Lane 3 shows purified recombinant Epigen running at ~7 kDa by Coomassie staining on an SDS-PAGE gel. G, glycosylation sites; AP, active peptide; TM, transmembrane domain; WB, Western blot; C, Coomassie stain.

Epigen Stimulation Stimulates Phosphorylation of MAP Kinase—We assessed whether Epigen, like other EGF family members, could activate the Ras/MAP kinase/c-fos signal transduction pathway (26). For this purpose, primers were designed to amplify cDNA that encoded Epigen from residues Leu53 to Ala103. Polymerase chain reaction products were subcloned into the bacterial expression vector pET16b, in frame with an N-terminal poly-histidine tag (Fig. 3A). The presence of a poly-histidine tag at a similar position in TGFα was found to have no effect on its biological activity (19). The expression vector was transformed into Escherichia coli to prepare recombinant Epigen (Fig. 3B). We assayed for MAP kinase phosphorylation upon stimulation of HaCaT cells by Epigen over a 20-min period. In this assay, phosphorylated MAP kinase was detected by Western blot analysis using an antibody that recognizes the phosphorylated forms of MAP kinase only (Fig. 4, A–C, panel P). As shown in Fig. 4A, an increase in MAP kinase phosphorylation was detected following a 5-min incubation of HaCaT cells with Epigen. The level of MAP kinase phosphorylation steadily increased to a maximal level at 20 min of stimulation. TGFα also induced MAP kinase phosphorylation after 5 min of incubation; however, maximal levels were reached at 10 to 15 min post-stimulation and had begun to decrease by 20 min (Fig. 4B). A control protein was only able to stimulate weak MAP kinase phosphorylation at 20 min when compared with Epigen and TGFα (Fig. 4C). Reprobing each blot with an antibody recognizing total MAP kinase verified that an equal quantity of protein from the control and test samples had been analyzed (Fig. 4, A–C, panel T).

Epigen Activates Genes under the Control of the SRE—We then assessed whether Epigen activated genes under the control of the SRE. Reporter constructs containing concatamerized SRE sequences upstream of a luciferase gene were stably transfected into HaCaT cells as described under “Experimental Procedures.” Reporter activity was evaluated by measuring luciferase levels. As shown in Fig. 5A, a dose-dependent increase in luciferase level was detected when Epigen was added to the stably transfected HaCaT cell line. A 3-fold increase in luciferase levels was observed at the highest Epigen concentration used, with luciferase levels returning to baseline at 1.8 nM. TGFα stimulated a 5-fold increase in luciferase levels at 18 nM, with luciferase levels decreasing to background at 2.0 × 10⁻² nM (Fig. 5B). EGF was most potent in this assay, promoting a 4.5-fold increase in luciferase levels at 1.8 nM (Fig. 5C). Luciferase levels induced by this ligand had not returned to baseline even at concentrations of 2 × 10⁻² nM. These results suggest that recombinant Epigen activates a signaling pathway through which EGF and TGFα exert their biological effects, consistent with activation of c-erbB-1 or other members of the c-erbB family.

Epigen Is Mitogenic for Epithelial Cells—To further examine whether Epigen displays EGF and TGFα-like activities, HaCaT cells were assayed for their proliferative responses to Epigen. As shown in Fig. 6A, Epigen promoted the growth of HaCaT cells in a dose-dependent manner. Maximal growth was obtained at a concentration of 1.75 nM. To compare Epigen proliferation with that promoted by other EGF family members, EGF and TGFα were also used to stimulate HaCaT cells. EGF was the most potent growth factor and stimulated maximal growth at 0.01 nM (Fig. 6C), whereas maximal growth by TGFα was obtained at a 10-fold higher concentration of 0.1 nM (Fig. 6B).

c-erbB-1 Is Activated upon Epigen Stimulation—To determine whether c-erbB-1 played a role in the mitogenic responses obtained, a blocking antibody to this receptor was added concurrently with a constant amount of Epigen or TGFα in a growth assay. We found that addition of anti-c-erbB-1 reduced the mitogenic effects of Epigen by up to 60% at antibody concentrations of 250 ng/ml (Fig. 7A). The mitogenic effects of TGFα were reduced by up to 50% at antibody concentrations of 500 ng/ml (Fig. 7B). These results suggested that c-erbB-1 was
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RESULTS

Results are representative of at least three separate experiments.

**Experimental Procedures.** The mean and S.D. were calculated from three separate wells and are represented as fold-induction of the reporter gene relative to control. Results are representative of at least three separate experiments.

**Figure 5. Induction of genes under the control of the SRE.** HaCaT cells containing concatamerized SRE sequences coupled to a luciferase reporter gene were stimulated with various concentrations of Epigen (A), TGFα (B), or EGF (C) for 6 h as described under “Experimental Procedures.” The mean and S.D. were calculated from three separate wells and are represented as fold-induction of the reporter gene relative to control. Results are representative of at least three separate experiments.

**Figure 6. Induction of HaCaT growth by Epigen (A), TGFα (B), or EGF (C).** HaCaT cells were stimulated with various concentrations of each ligand for 5 days in low serum conditions as described under “Experimental Procedures.” The mean and S.D. were calculated from three separate wells. Background 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) levels for this assay are shown in A. Results are representative of at least three separate experiments.

DISCUSSION

Here we report the molecular cloning, expression, and biochemical activity of Epigen, a novel member of the EGF ligand superfamily. Epigen was identified from a cDNA library constructed from a population of immature keratinocytes due to its conditional activity of Epigen, a novel member of the EGF ligand superfamily. Epigen conforms to the characteristics described above and thus is likely to exhibit EGF- and TGFα-like structural and receptor binding properties.

All EGF superfamily members identified to date that bind to c-erbB-1 have been isolated as small, secreted peptides from culture supernatants. Subsequent analysis of the mRNA from which they are encoded indicated that all are derived from membrane-bound precursors that are proteolytically cleaved from the plasma membrane (9, 11–16). The protein sequence of Epigen has a putative transmembrane domain situated in an region of an EGF superfamily member that is absolutely conserved across the entire superfamily (11). At the amino acid level, Epigen displays all the homology with epiregulin, the latest member of the EGF superfamily (11). However, EGF family members can be distinguished from proteins that do not display EGF-like activities (e.g., see Ref. 54). However, EGF family members can be distinguished from these proteins due to the strict conservation of one glycine and one arginine residue within this unit. The arginine residue is absolutely required for strong binding to c-erbB-1, as shown by extensive point mutation analysis of TGFα (18). Epigen conforms to the characteristics described above and thus is likely to exhibit EGF- and TGFα-like structural and receptor binding properties.

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probably involved in the transmission of recombinant Epigen mitogenic activity. To further examine the involvement of c-erbB-1 in Epigen-driven growth, we assayed for c-erbB-1 phosphorylation upon stimulation of A431 cells by Epigen and TGFα. In this assay, phosphorylated c-erbB-1 was detected by Western blot analysis using an antibody that recognizes the phosphorylated forms of c-erbB-1 only. As shown in Fig. 7C, an increase in c-erbB-1 phosphorylation was detected following a 15-min incubation of A431 cells with Epigen or TGFα. A control protein was not able to induce c-erbB-1 phosphorylation during this time. Reprobing each blot with an antibody recognizing total c-erbB-1 protein verified that an equal quantity of protein from the control and test samples had been analyzed.

**Figure 7. Inhibition of Epigen- or TGFα-induced HaCaT growth by an antibody to c-erbB-1 and induction of c-erbB-1 phosphorylation by Epigen and TGFα on A431 cells.** HaCaT cells were grown in 1.75 nM Epigen (A) or 0.1 nM TGFα (B) in the presence of various concentrations of anti-c-erbB-1 antibody (closed squares) or control antibody (open squares). The mean and S.D. were calculated from three separate wells, and results are represented as percent growth relative to the isotype control antibody. Results are representative of at least three separate experiments. C, A431 cells were stimulated with 18 nM Epigen, TGFα, or control protein at 37 °C for 15 min. Cells were processed as described under “Experimental Procedures.” Lysates were analyzed by SDS-PAGE and Western blotting using antibodies to the phosphorylated form of c-erbB-1 protein or to total c-erbB-1.

**Figure 5. Induction of genes under the control of the SRE.** HaCaT cells containing concatamerized SRE sequences coupled to a luciferase reporter gene were stimulated with various concentrations of Epigen (A), TGFα (B), or EGF (C) for 6 h as described under “Experimental Procedures.” The mean and S.D. were calculated from three separate wells and are represented as fold-induction of the reporter gene relative to control. Results are representative of at least three separate experiments.

**Figure 6. Induction of HaCaT growth by Epigen (A), TGFα (B), or EGF (C).** HaCaT cells were stimulated with various concentrations of each ligand for 5 days in low serum conditions as described under “Experimental Procedures.” The mean and S.D. were calculated from three separate wells. Background 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) levels for this assay are shown in A. Results are representative of at least three separate experiments.
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identical position to that of the other EGF superfamily members. This suggests that Epigen is also synthesized as a transmembrane-tethered precursor, from which an active peptide is cleaved. However, because the TGFα precursor is capable of binding to, and inducing phosphorylation of, c-erbB-1 while situated on the plasma membrane (55, 56), it is possible that the transmembrane-tethered form of Epigen is similarly active.

In silico analysis of the keratinocyte library from which Epigen was identified indicated that expressed sequence tags encoding Epigen were present at a frequency of less than 1:10,000 sequences. Northern blot analysis of adult mouse tissues confirmed that the mRNA for Epigen was present at low levels in testis, heart, and liver tissues. The expression profiles of several EGF superfamily members overlap with the profile obtained for Epigen. For example, HB-EGF is also found predominantly in lung, brain, and heart tissues, although also in skeletal muscle, kidney, and spleen (15). Epiregulin is present in heart, lung, and smooth muscle tissues of the adult mouse. TGFα is present in brain, but is also present in pituitary glands and macrophage cells (11, 33, 52, 57). Therefore, although other EGF superfamily members are expressed in tissues also expressing Epigen, it appears that the combined tissue distribution profile and expression level of each family member is unique.

Recombinant Epigen was purified from a bacterial expression system and used in several assays designed to examine whether it behaved as a classical EGF family member in vitro. The transformed epithelial cell line HaCaT was chosen as a model system because it is very sensitive to the effects of EGF and TGFα. Epigen and TGFα both induced phosphorylation of MAP kinase in HaCaT cells over a 20-min stimulation period. The magnitude of MAP kinase phosphorylation induced by both ligands was similar; however, TGFα-induced phosphorylation had reached maximal levels at 10 min and was sustained at this level, whereas Epigen-induced phosphorylation reached maximal levels at 20 min. Phosphorylation of MAP kinases often results in the transcription of many genes that are under the control of a common regulatory element, the SRE. A reporter gene linked to upstream SRE enhancer elements was induced in a dose-dependent manner by Epigen, TGFα, and EGF. Epigen induced a 3-fold increase in reporter activity at the highest concentration tested, whereas TGFα and EGF induced a 5- and 4.5-fold increase, respectively, in reporter activity at optimal concentrations. Thus, the profile of MAP kinase phosphorylation and SRE activation induced by Epigen, TGFα, and EGF was unique to each ligand, with EGF the most potent, and Epigen the least potent.

To examine how the signal transduction patterns observed with Epigen, TGFα, and EGF were translated into more specific cellular responses, we compared the induction of HaCaT growth by Epigen with the two known EGF superfamily members. All three proteins invoked a dose-dependent growth response. Epigen and TGFα promoted similar levels of growth at their optimum concentration, whereas EGF-driven growth was more potent. The transformed epithelial cell line HaCaT was chosen as a model system and used in several assays designed to examine specific cellular responses, we compared the induction of HaCaT growth by Epigen with the two known EGF superfamily members. All three proteins invoked a dose-dependent growth response. Epigen and TGFα promoted similar levels of growth at their optimum concentration, whereas EGF-driven growth was more potent. However, the optimal EGF concentration needed to promote growth of HaCaT cells was 10-fold less than that of TGFα, and the optimal concentration of Epigen was 10-fold greater than that of TGFα. An antibody to the extracellular domain of c-erbB-1 efficiently blocked the growth stimulation of HaCaT cells by Epigen and by TGFα, which suggested that c-erbB-1 was involved in the Epigen signaling pathway. Further evidence for the involvement of c-erbB-1 was obtained when Epigen was found to induce phosphorylation of c-erbB-1 in A431 cells; this response was somewhat weaker than that induced by TGFα. Therefore, although c-erbB-1 is clearly activated during Epigen signaling, this activation may be a secondary event following the binding of Epigen to another receptor.

A detailed examination of Epigen binding to c-erbB-1 and the other erbB receptors is necessary to determine which is the primary receptor for Epigen.

Differences in the duration and intensity of MAP kinase phosphorylation and proliferative responses are well documented for EGF superfamily members (8, 30, 32, 33). In NIH/3T3 cells, epiregulin is more potent than EGF at higher concentrations, whereas at low concentrations, EGF is more potent (33). In smooth muscle cell growth, HB-EGF is significantly more potent than EGF at all concentrations (8). These differences can often be correlated with the affinity of each ligand for the c-erbB receptors on specific cells, such that when compared with EGF, HB-EGF has greater affinity for c-erbB-1 on smooth muscle cells, and epiregulin has a lower affinity for c-erbB-1 on NIH/3T3 cells (8, 23, 32, 33). These features are thought to allow for an enormous range and control of signal output (23). Therefore it may be that the affinity of Epigen for c-erbB-1 on HaCaT cells at least partially accounts for the signal transduction patterns and growth responses obtained. Alternatively, because Epigen was identified by molecular, rather than biochemical, means, it is possible that native Epigen has a slightly different amino acid composition at the N or C terminus from that of recombinant Epigen. C-terminal residues in particular are known to have a significant influence on receptor binding, because a C-terminally extended recombinant AR protein was found to be 10 times more active than truncated forms (58). The isolation of native Epigen from culture supernatants will address these issues.

We have identified an expressed sequence tag that encodes Epigen, a novel member of the EGF superfamily of peptide growth factors. To our knowledge, this is the first example of an EGF-like ligand to be identified by expressed sequence tag data base screening. Epigen displays all the structural characteristics of an EGF family member and behaves similarly to TGFα and EGF in signal transduction and growth assays. However, there are ligand-specific differences in magnitude of growth response and concentration of ligand giving maximal response in all the assays performed. Other cross-comparative assays show that EGF superfamily members are able to induce their own mRNA production and that of other family members in ligand-specific patterns (59–61). This suggests that Epigen and the other members of the EGF family have distinct, non-redundant functions. Members of the EGF family have a number of overlapping functions in vitro, and this has prompted increased analysis of the contribution each ligand makes to growth, development, and disease. For example, triple knockout mice bearing mutations in TGFα, Epiregulin, and AR have recently been created, uncovering a role for AR in mammary ductal morphogenesis (62). Current investigations in this laboratory are focused on uncovering a specific in vivo role for Epigen in normal and aberrant growth and development.

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