Auto- and Cross-induction within the Mammalian Epidermal Growth Factor-related Peptide Family*

(Received for publication, May 31, 1994)


From the Department of Pediatrics, Division of Gastroenterology, ‡‡Department of Cell Biology, and ¶¶Department of Medicine, Division of Gastroenterology, Vanderbilt University School of Medicine, Nashville Tennessee 37232-2576, the ¶¶Veterans Affairs Medical Center, Nashville, Tennessee 37232, (!Scisio Nova, Inc., Mountain View, California 94043, and ¶¶¶Department of Dermatology and Biochemistry and Molecular Biology, Mayo Clinic Foundation, Rochester, Minnesota 55902.

Several polypeptide growth factors related to epidermal growth factor (EGF) have been identified recently, including transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), and betacellulin (BTC). These peptides all bind to the EGF receptor (EGFR). In an effort to understand redundancy within this peptide family and interactions among these related peptides, we compared the biological activities of EGF, TGF-α, AR, and HB-EGF in an EGF-responsive, nontransformed intestinal epithelial line (RIE-1) and also determined the effect of individual EGF-related peptides on the expression of related family members in these cells. TGF-α, AR, HB-EGF, and EGF were equipotent in stimulating [3H]thymidine incorporation by RIE-1 cells and bound the EGFR with equivalent affinity. Each EGF-related peptide induced the mRNA expression of the remaining family members, including BTC. HB-EGF and AR mRNAs were induced rapidly (within 30 min) and to a greater extent than TGF-α and BTC mRNAs, suggesting heterogeneity in the molecular mechanisms for induction. This same pattern was observed for all EGF-related peptides tested. A similar pattern of mRNA induction was observed in secondary cultures of human keratinocytes and in LIM1215 colon adenocarcinoma cells. Nuclear run-on analysis showed that induction of AR and HB-EGF is, at least in part, regulated at the level of gene transcription. Concurrent treatment with HB-EGF and cycloheximide resulted in superinduction of HB-EGF and AR, suggesting that these peptides are immediate early genes in RIE-1 cells. Our results demonstrate an equivalent biological response to EGF-related peptides in RIE-1 cells and further indicate that extensive auto-induction and cross-induction occur within the EGF-related peptide family in several EGF-responsive epithelial cell types.

The number of mammalian polypeptide growth factors exhibiting significant sequence identity with epidermal growth factor (EGF) has increased dramatically in recent years (reviewed in Ref. 1). EGF, transforming growth factor-α (TGF-α) (2), amphiregulin (AR) (3), heparin-binding EGF-like growth factor (HB-EGF) (4), and betacellulin (BTC) (5) share at least 28% sequence identity and 100% conservation of the 6 cysteine residue positions in the mature peptide sequence. All are synthesized as glycosylated integral membrane precursor proteins with extracellular domains that contain an EGF-like mature peptide sequence. Although cripto contains a cysteine-rich EGF-like domain and has been considered a candidate EGF family member (6); a recent report clarifies this issue by concluding that cripto does not bind the EGF receptor (EGFR) and thus does not belong to the EGF-related peptide family (7). The membrane-associated precursors of EGF and TGF-α are biologically active (8, 9), as are the mature, soluble peptides that are released from the extracellular domain by proteolytic cleavage (10). The biological activity of the transmembrane forms of AR, HB-EGF, and BTC has not been investigated.

All the EGF-related peptides bind to the 170-kilodalton cell surface membrane receptor, the EGFR. Inasmuch as the protein structure is conserved and a common receptor is utilized, it is not surprising that EGF-related peptides share a spectrum of biological activities, the most widely appreciated of which is stimulation of cellular growth. In fact, qualitative differences in function have not been described suggesting that a great deal of functional redundancy must exist within the EGF family. Several quantitative differences in biological activity have been delineated for the two best studied family members, EGF and TGF-α. For example, TGF-α is more potent than EGF in stimulation of calcium release from fetal long bones (11), induction of angiogenesis (12), and in stimulation of blood flow (13). However, the relative potencies of newer family members such as AR and HB-EGF in these and more traditional growth assays have not yet been well characterized. Despite the widespread distribution of TGF-α and EGFR in tissues of normal mice, recent studies have shown that disruption of the normal TGF-α gene by homologous recombination results in relatively minor phenotypic alterations including wavy hair and curly whiskers, which occur because of disorganization of hair follicle structure. Less frequently, ocular abnormalities were observed (14, 15). Similar "minimal phenotypes" have been observed when other genes of presumed importance have been disrupted (16, 17), leading to the hypothesis that redundancy in protein families results in replacement of a missing function. Taken together,

1 The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; AR, amphiregulin; HB, heparin-binding; BTC, betacellulin; HK, human keratinocytes; RIA, radioimmunoassay.

22817
these observations suggest that analysis of the biological complexity of the EGF family will require investigation of individual as well as integrated EGF-related protein function.

Increased TGF-α mRNA and protein expression occurs following treatment of various epithelial cell lines with TGF-α (18, 19), a phenomenon that has been termed “auto-induction.” Auto-induction has also been observed for other factors including c-sis (the B chain of platelet-derived growth factor) (20), the three mammalian isoforms of TGF-β (21, 22), and interleukin 1 (23). The biological relevance of auto-induction remains uncertain, but it may represent a mechanism by which an initial growth factor signal is amplified and sustained. In addition to auto-induction, induction of TGF-α by EGF (18), and induction of TGF-β1, TGF-β2, and TGF-β3 isoforms by TGF-β1 and TGF-β2, have been reported (22). Collectively, these observations suggest that appreciation of the integrated activities of a growth factor family will require analysis of the effect of individual growth factors on the expression of related factors within the same family. Herein, we describe the relative potency of EGF, TGF-α, AR, and HB-EGF in proliferating a nontransformed rat intestinal epithelial cell line and report extensive inter-regulation of EGF-related polypeptide growth factor gene expression by these same factors in several epithelial cell types. The results also indicate that both transcriptional and post-transcriptional mechanisms are responsible for regulation among family members.

MATERIALS AND METHODS

Cell Lines and Reagents—The rat intestinal epithelial (RIE-1) cell line was obtained from Dr. Kenneth Brown (Cambridge, UK). RIE-1 cells are a diploid, nontransformed epithelial line isolated from rat small intestine (24). The cells are grown in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum (J. R. Scientific Co., Woodland, CA). Human keratinocytes (HK) were derived from human newborn foreskin and cultured in serum-free MCD 153 containing EGF, insulin, and bovine pituitary extract as well as ethanolamine, phosphoethanolamine, and hydrocortisone (25). LIM1215 (human colon adenocarcinoma cells) were maintained in McCoy’s medium 5A supplemented with 10% fetal bovine serum. Prior studies indicated that these three lines are responsive to EGF (26–28). All assays were done before passage 20. Recombinant human EGF was purchased from Amgen (Thousand Oaks, CA) and recombinant human TGF-α was kindly supplied by Dr. Rik Derynck (University of California at San Francisco). Recombinant amphiregulin was kindly provided by Greg Plowman (Bristol-Myers Squibb Pharmaceutical Research Institute). Recombinant human HB-EGF was prepared by Sciocs Nova Inc. (Mountain View, CA) and included an initiating methionine followed by residues 73–149 of the HB-EGF precursor (4).

Mitogenic Assays—RIE-1 cells were seeded in 24-well plates. Cells were placed in serum-free medium for 72 h as soon as confluence was reached. Preliminary [3H]thymidine experiments were conducted to determine that quiescence was reached 72 h after serum deprivation and that uptake was maximal 18 h after stimulation with EGF. Cells were then restimulated with the indicated growth factor in the absence of serum and labeled with 1 μCi/ml [3H]thymidine (50–80 Ci/mmol, Du Pont NEN), between the 18th and 20th h after treatment. Radioactivity incorporated into trichloroacetic acid-insoluble material was determined by scintillation counting. All experiments were performed in triplicate or quadruplicate and repeated at least once.

Competitive Binding Assays—RIE-1 cells were cultured in 24-well plates until near confluence. Fresh medium was added 24 h before the assay. Cells were washed for 1 h in binding buffer (128 mM NaCl, 5 mM KCl, 5 mM MgSO4, 1.2 mM CaCl2, 50 mM HEPES, pH 7.5, and 0.1% bovine serum albumin). Cells were incubated for 2 h at room temperature with 125I-labeled TGF-α and displacing ligands at concentrations ranging from 10−7 to 10−12 M. The cells were then washed three times with ice-cold binding buffer and solubilized in 1% Triton X-100 for 30 min. Cell-associated radioactivity was determined in a γ-counter. Duplicate experiments were performed and results are the mean ± standard deviation of six data points.

Isolation of Poly(A) RNA and Northern Blot Analysis—Total cellular RNA was extracted by the method described by Schwab et al. (29).

Fig. 1. Stimulation of RIE-1 thymidine incorporation by EGF-related peptides. Confluent RIE-1 cells in 24-well plates were placed in serum-free Dulbecco’s modified Eagle’s medium for 72 h. These quiescent cells were then stimulated (in the absence of serum) for 18 h with the indicated concentrations of EGF-related peptides. Thymidine uptake was determined by a 2-h pulse between h 18 and 20. Radioactivity incorporated into trichloroacetic acid insoluble material was determined by scintillation counting. All experiments were performed in triplicate or quadruplicate and repeated at least once.

Oligo(dT)-selected RNA was separated by electrophoresis in 1.2% agarose, formaldehyde gels, and Northern blotting was performed as described previously (30, 31). Hybridizations with species-specific probes labeled by RNA polymerase-directed reverse transcription (EGF, TGF-α, AR, and BTC) or random primer extension (HB-EGF) were performed under conditions described previously (32, 33). The probe designated 1B15 is used as a constitutive sequence and has been described previously (34, 35).

Isolation of Conditioned Medium and Preparation of Cell Lysates for TGF-α and AR Radioimmunoassay (RIA)—RIE-1 cells were grown to near-confluence in 6-well plates, washed in isotonic buffer twice, and placed in serum-free Dulbecco’s modified Eagle’s medium for 72 h. The medium was removed, and the cells were washed twice with isotonic buffer and fed with serum-free medium containing 3 μM HB-EGF. Medium was collected immediately (control) and 4, 8, and 24 h after treatment. The cells were collected into 25 μM Tris-HCl (pH 8.0), 60 μM NaCl, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40. The rat TGF-α antibody used for RIA was developed in collaboration with East Acres Biologicals (Southbridge, MA). The RIA has been described in detail (36) and is highly sensitive and specific for rat TGF-α. Human TGF-α, rat EGF, and human AR are not recognized by this antibody. The AR antibody and RIA have been described in detail previously (37).

Briefly, the antibody was developed in rabbits to residues 21–46 of mature human AR. The AR RIA does not detect EGF or TGF-α and is sensitive to AR concentrations as low as 10 pg/ml.

Nuclear Run-on Transcription Assay—RIE-1 nuclei were isolated from quiescent cells and cells treated with TGF-α (3 μM) for various intervals. Nuclei were prepared in Nonidet P-40 lysis buffer (10 μM Tris-HCl, pH 7.4, 10 μM NaCl, 3 μM MgCl2, and 0.5% Nonidet P-40). Nuclear run-on transcripts were labeled as described (38). Equivalent amounts of radioactivity were added to each nucleotidylate filter and hybridized for 4 days at 42 °C in the presence of 50% formamide. Filters were prepared by blotting 5.0 μg of plasmid DNA containing either TGF-α, AR, HB-EGF, BTC, or 1B15 cDNA inserts. Post-hybridization washes were performed at 42 °C using 0.1 x SSC, 0.1% SDS, and 1 μM EDTA, and autoradiograms were prepared.

RESULTS

Mitogenic Assays—RIE-1 proliferation was significantly stimulated by EGF, TGF-α, AR, and HB-EGF (Fig. 1). All were equipotent in stimulating RIE-1 DNA synthesis. Stimulation occurred at a concentration between 10−10 and 10−2 M, with maximal stimulation occurring at approximately 10−4 M. This dose response is similar to that observed in other epithelial prolifera-

Isolation of Poly(A) RNA and Northern Blot Analysis—Total cellular RNA was extracted by the method described by Schwab et al. (29).
Induction of Multiple Family Members by Treatment with 3 nm EGF, TGF-α, HB-EGF, or AR—Poly(A) RNA was isolated from confluent RIE-1 cells at various times over a 24-h interval following stimulation of quiescent cells with EGF, TGF-α, AR, or HB-EGF. Northern blots were prepared with murine specific probes complementary to EGF, TGF-α, AR, HB-EGF, BTC, and a constitutive probe, 1B15. Results of TGF-α treatment and HB-EGF treatment are shown in Figs. 3 and 4 and reflect those observed for EGF and AR. EGF transcripts were not detected at any time point, under any condition (data not shown). TGF-α expression was not detectable in quiescent cells. A low level of expression was seen beginning 1–2 h after treatment, and maximal TGF-α expression was detected 4–12 h after treatment in all cases. The kinetics of induction were similar to those reported for TGF-α-treated keratinocytes and colon carcinoma cells (18, 19), even though the magnitude of induction was less in the present study. In contrast to the induction of TGF-α mRNA, the magnitude of HB-EGF and AR induction was more pronounced. Furthermore, induction of HB-EGF and AR was noted within 30 min of stimulation and peaked 1–2 h after treatment. BTC mRNA was induced 1 h after treatment and was maximal 2–4 h after treatment. The magnitude of BTC induction was similar to that of TGF-α and less than that reported for HB-EGF and AR.

The induction of EGF-related peptide mRNAs was also investigated in two additional EGF-responsive cells, HK (Fig. 5A) and LIM1215 (Fig. 5B). Although all EGF-related peptides were not tested in these two cell types, a pattern of induction strikingly similar to that observed in RIE-1 cells occurred after treatment of HK with both 3 nm AR (Fig. 5A) and 3 nm HB-EGF (data not shown) and treatment of LIM1215 cells with TGF-α (3 nm). Slight differences in the pattern of induction were noted among the three cell types. For example, HK cells continued to express low levels of EGF-related peptide mRNA even after 72 h at confluence in growth factor-free medium, a finding that obscured somewhat the degree of mRNA induction. Interestingly, the expression of AR in LIM1215, a transformed line, increased linearly during the 24 h of AR treatment, again supporting alterations in the pattern of inductive events between nontransformed and transformed cells, as demonstrated previously (19). Furthermore, under the conditions of these experiments, BTC expression was not detected in either HK or LIM1215 cells. Despite these small differences, a clear induction of the various EGF-related peptide mRNAs occurred in all three cell types, and a differential response in the kinetics of induction was preserved.

The mechanism(s) by which EGF-related peptides are induced was examined by treatment of quiescent RIE-1 cells with HB-EGF for 2 h in the presence of actinomycin D or cycloheximide. As expected, HB-EGF mRNA levels increased following HB-EGF treatment. Inhibition of protein synthesis by cycloheximide resulted in a superinduction of HB-EGF mRNA as shown in Fig. 6. Treatment with cycloheximide alone also resulted in induction of HB-EGF mRNA. Inhibition of RNA synthesis by actinomycin D abrogated the induction of HB-EGF by HB-EGF, and actinomycin D exposure alone had the same effect suggesting that the mRNA has a short half-life. These results suggest the presence in RIE-1 cells of a labile activity which stabilizes the HB-EGF mRNA. Similar conclusions could not be made regarding the induction of TGF-α, since under the conditions of this assay, TGF-α mRNA was not detectable during the first 2-h interval after treatment.

Nuclear Run-on Assays—The mechanisms by which EGF-related mRNAs are induced were further investigated by nuclear run-on transcription assays. Fig. 7 shows that treatment of quiescent RIE cells with 3 nm TGF-α did not result in significantly increased rates of TGF-α or BTC transcription, while induction of HB-EGF and AR transcription increased 30 and 60 min after treatment before returning to normal. These results indicate the existence of both transcriptional and post-transcriptional pathways for auto-induction and cross-induction within the mammalian EGF family and heterogeneity in the level of regulation depending on the mRNA species being induced.
sequences as described under "Materials and Methods." Northern blots were prepared and probed with the indicated "P-labeled RNA were loaded per lane.

TGF-a for the intervals shown. Poly(A) RNA was isolated and Northern of HB-EGF treatment and reached a maximum of 2.2-fold cell-associated immunoactivity and no increase in conditioned remained low for the 24 h duration of the assay.

Effects of actinomycin D and cycloheximide on HB-EGF auto-induction. Quiescent HK cells were treated with 3 nm AR and quiescent LIM1215 cells were treated with 3 nm TGF-a for the intervals shown. Poly(A) RNA was isolated and Northern blots were prepared and probed with the indicated "P-labeled sequences as described under "Materials and Methods." Two µg of poly(A) RNA were loaded per lane.

Fig. 5. Induction of the mRNA for EGF-related peptides in HK cells (A) and LIM1215 cells (B). Quiescent HK cells were treated with 3 nm AR and quiescent LIM1215 cells were treated with 3 nm TGF-a for the intervals shown. Poly(A) RNA was isolated and Northern blots were prepared and probed with the indicated "P-labeled sequences as described under "Materials and Methods." Two µg of poly(A) RNA were loaded per lane.

Discussion
The discovery of each new EGF-related peptide adds additional complexity to understanding the integrated biological function of the EGF family. In the present study, we have examined the biological activity of four members of the EGF family and also determined the extent to which these same four polypeptides influence the expression of the other related peptides within the EGF family. We find uniformity in the proliferative response to EGF-related peptides in the nontransformed intestinal epithelial line RIE-1. In other reports, an enhanced response to HB-EGF relative to EGF has been noted; for example, 100 pg/ml HB-EGF and 4 ng/ml EGF stimulated smooth muscle cell growth to the same extent, an observation which may be explained in part by the greater affinity of HB-EGF for the EGFr (4). We found that HB-EGF competed as effectively as other EGF-related peptides for 125I-labeled TGF-a binding to RIE-1 cells. It is not known if increased potency of HB-EGF relative to other family members is a common phenomenon or restricted to selected cell lineages such as smooth muscle cells. AR also appeared equivalent to EGF and TGF-a in the stimulation of mitogenesis. This contrasts somewhat with observations made in the GEO cells, a colon carcinoma cell line that is 5-fold less responsive to purified AR than EGF (39). Inasmuch as recombinant preparations of AR are not yet widely available, these observations are likely due to heterogeneity in the biological activity of purified preparations of AR. Taken together, these observations indicate that no significant differences exist within the EGF family with respect to stimulation of mitogenesis in RIE-1 cells, although one cannot exclude that differences may exist in vivo or under cell culture conditions other than those used in the assays described herein. It is plausible that glycosaminoglycan and extracellular binding protein (such as heparin and heparan sulfate proteoglycans) may alter the affinity of some of the EGF-related peptides for receptor binding motifs, or that differences in post-receptor processing may contribute to heterogeneity in the biological response observed under certain experimental conditions.

Previous work (18) demonstrated up-regulation of TGF-a mRNA and protein levels by EGF and auto-induction of TGF-a gene expression in cultured keratinocytes. The present study expands these observations by showing that induction of an individual family member by other EGF-related peptides is a more generalized process in epithelial cells. While the precise molecular mechanism(s) by which this induction of EGF-related peptides occurs is not well understood, a prior study suggested that TGF-a auto-induction is complex (19). For example, TGF-a auto-induction in nontransformed human keratinocytes occurred primarily by prolongation of mRNA half-life, while TGF-a auto-induction in a transformed colon epithelial line (LIM1215) occurred by a transcriptional mechanism. Auto-induction in both lines was partially inhibited by blockade of the protein kinase C pathway (19).

In the present study, induction of TGF-a and BTC appeared to occur post-transcriptionally, although it must be considered...
that the magnitude of increase in transcription of these two genes was below the level of detection by nuclear run-on transcription assay. The absence of a transcriptional level of control for the induction of TGF-α by TGF-α in the nontransformed RIE-1 line supports the previously reported observations in nontransformed keratinocytes cited above (19). In contrast, the induction of HB-EGF and AR occurred, at least in part, at the level of transcription. The observation that HB-EGF and AR are induced more rapidly and to a relatively greater level (Figs. 2 and 3) than TGF-α and BTC may be a reflection of the differences in the mechanisms of induction reported herein. Identification of regulatory 5′-flanking sequences in EGF-related genes that mediate the transcriptional component of these inductive events would be of interest, particularly since the rat TGF-α promoter is notable for the paucity of recognizable promoter elements such as a TPA-responsive element and a TATA box (40, 41). A proximal 313 bp in the human TGF-α 5′-flanking sequence that mediates the responsiveness to EGF and phorbol esters has been identified by Raja and co-workers (42), but this region did not contain an AP-2 binding site. An estrogen-responsive region has also been described in the 5′-flanking sequence (43). The precise sequences responsible for the molecular regulation of TGF-α and other EGF-related peptides by members of the same family must await complete sequencing and comparative analysis of the promoter regions of each family member.

Our studies show that HB-EGF and AR are rapidly induced by EGF-related peptide treatment. Induction occurs by a transcriptional mechanism and mRNA levels are superinduced by auto-induction and cross-induction within the EGF-related factor family. Further investigation of both cis- and trans-acting factors responsible for auto-induction and ultimately contribute to a more complete understanding of the integrated biological functions of the EGF family of growth factors.

Acknowledgments—Dr. Judith Abraham and Deborah Damm kindly provided the rat HB-EGF clone. Peter Strathsbur purified the recombinant HB-EGF. Dr. Doug Hanahan kindly provided the betacellulin probe.

REFERENCES


Auto- and cross-induction within the mammalian epidermal growth factor-related peptide family.

J A Barnard, R Graves-Deal, M R Pittelkow, R DuBois, P Cook, G W Ramsey, P R Bishop, L Damstrup and R J Coffey


Access the most updated version of this article at http://www.jbc.org/content/269/36/22817

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/269/36/22817.full.html#ref-list-1