Regulation of Epidermal Growth Factor Receptor Gene Expression by Retinoic Acid and Epidermal Growth Factor

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In normal rat kidney fibroblasts, retinoic acid increases the level of epidermal growth factor (EGF) binding and synergizes with EGF and transforming growth factor-β to stimulate anchorage-independent growth. We now demonstrate that retinoids act by increasing the rate of transcription of the EGF receptor gene, resulting in elevated mRNA levels. No effect of retinoic acid on EGF receptor mRNA half-life, measured after actinomycin D treatment, was observed. In the same system, EGF was also able to increase expression of its own receptor through an elevation in mRNA levels. These effects were specific since retinoids and EGF did not alter transcript levels for fibronectin, α-tubulin, or β2-microglobulin. These results demonstrate that the EGF receptor gene is a target for regulation by multiple growth-stimulating factors.

The membrane receptor for the polypeptide mitogen epidermal growth factor (EGF) is a 170-kDa tyrosine kinase whose activity and expression are regulated by multiple mechanisms (1). Binding of EGF to its receptor leads to an increase in receptor autophosphorylation and receptor enzymatic activity, followed by rapid internalization and degradation of the ligand-receptor complex. Subsequent to EGF treatment, a compensatory increase in EGF receptor synthesis has been observed in several epithelial cell lines resulting from an elevation in EGF receptor mRNA levels (2-4). Heterologous ligands also modulate EGF receptor expression through multiple loci. Activators of protein kinase C, such as phorbol esters and platelet-derived growth factor, are able to inhibit high affinity EGF binding and EGF stimulation of the tyrosine kinase activity of the EGF receptor via phosphorylation of the cytoplasmic domain (1). Certain regulators of differentiation, such as retinoic acid, estrogen, and transforming growth factor-β (TGF-β), increase EGF binding capacity in select cell systems (5-7). We have recently demonstrated that TGF-β enhances the rate of transcription of the EGF receptor gene in NRK cells (8). Estrogen and retinoic acid may also regulate (directly or indirectly) the EGF receptor at the level of gene expression since it has been shown that the intracellular receptors for these agents function as transcription factors (9-11).

The ability of retinoic acid to increase the level of cell-surface binding of EGF has been noted in numerous nondifferentiating cell systems (5, 12-14). This increase in EGF binding activity is specific to the retinoid analogs that exhibit activity in biological assays such as induction of teratocarcinoma cell differentiation (5). Several lines of evidence suggest that retinoids act by increasing the number of cell-surface EGF receptors without affecting receptor affinity. The effect of retinoic treatment on EGF binding (13-15), EGF internalization and degradation, and rate of recovery of EGF binding after down-regulation (16) suggests that the rate of EGF receptor synthesis is the target of retinoic acid action.

In this paper, we examine the effect of retinoic acid on EGF receptor gene expression in NRK fibroblasts. Retinoic acid acts synergistically in this system with TGF-β and EGF in the induction of anchorage-independent growth (12). Both TGF-β and retinoic acid increase the level of EGF binding to NRK cells. Like TGF-β, retinoic acid increases the rate of EGF receptor gene transcription in NRK cells.

EXPERIMENTAL PROCEDURES

Materials

Stock solutions of 1-5 mM all-trans-retinoic acid (Sigma) were made up in ethanol and stored at -20°C for up to 1 week. Manipulations with retinoic acid were performed under subdued light or yellow lamps. EGF (receptor-grade) was from Biomedical Technologies, Inc. (Stoughton, MA). 125I-Labeled EGF (150-200 μCi/μg) and radiolabeled nucleotides were from Du Pont-New England Nuclear. Actinomycin D was from Sigma.

Cell Culture

NRK cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum. Confluent cultures of NRK cells were made quiescent by incubation for 2 days in DMEM supplemented with insulin, transferrin, and selenium (ITS-Premix, Collaborative Research).

EGF Binding Assay

Cells were plated in DMEM plus 10% calf serum in 24-well dishes at least 2 days in advance of EGF binding determinations. Modulators or solvent (maximum final dilution of 0.1%) was added to the wells 1 day preceding determination of EGF binding levels.

For measurement of EGF binding levels, the cells were washed with binding medium (DMEM, 10 mM Hepes, pH 7.4, 0.5% bovine serum albumin). 125I-Labeled EGF, at concentrations between 5 and 80 pM, was added to 500 μl of binding medium in the wells. Unlabeled EGF, in greater than a 1000-fold excess over labeled EGF, was added to duplicate wells; and the dishes were incubated at 37°C for 60 min. After incubation, dishes were placed on ice and rapidly washed five times.
times with ice-cold binding medium. NaOH (0.2 N) was then added to the wells, and dishes were placed at 60-70 °C for 10 min or left overnight at room temperature. The solubilized cells were removed to counting tubes, the wells were washed with 0.2 N HCl, and the washes were pooled. Sample radioactivity, which was determined in duplicate, was quantitated in a Pharmacia LKB Biotechnology Inc. y-counter. Specific binding was determined by subtracting the amount of [32P]EGF bound to cells in the presence of excess unlabeled EGF from the total amount bound.

### RNA Isolation

Total RNA was isolated from NRK cells with guanidine isothiocyanate using the modified procedure described by Chirgwin et al. (17). Briefly, cells were lysed in GSCN (6 M guanidine hydrochloride, 2.5 M sodium citrate, pH 7.0, 0.5% sodium lauroylsarcosine, 0.1 M β-mercaptoethanol). The lysate was briefly sonicated and precipitated overnight at -20 °C after the addition of acetic acid to 25 mM and 0.75 volume of ethanol. After centrifugation at 7,500 g for 20 min at -10 °C, the resulting pellets were resuspended in 5 ml of GSCN and layered over a 1:1 cushion of 5.7 M CaCl2, 25 mM sodium acetate, pH 5.4. The RNA was pelleted by centrifugation in an SW 56 rotor at 34,000 rpm for 18-24 h. Pellets were resuspended in 10 mM Tris, 1% SDS, 10 mM EDTA with heating and precipitated with ethanol and sodium acetate at -20 °C. For short-term storage, RNA was kept in 95% ethanol at -70 °C. RNA samples were normalized for equal ribosomal RNA content, as determined by ethidium bromide staining of RNA electrophoresed on formaldehyde-agarose gels, before analysis. Poly(A)+ RNA was prepared by affinity chromatography with oligo(dT)-cellulose as described by Maniatis et al. (18), usually by batch absorption.

### Northern Blot Hybridization

RNA was electrophoresed in 0.9-1.0% formaldehyde-agarose gels by methods similar to those described in Maniatis et al. (18). After electrophoresis, the gel was exposed to short-wave ultraviolet light for 5 min to enhance transfer of large mRNAs. Transfer to nitrocellulose and subsequent baking were performed as described by Thomas (15). The filters were hybridized with 32P-labeled RNA probes were prehybridized for at least 3 h in 50% formamide, 0.8 M NaCl, 2.5 × Denhardt’s solution (50 × Denhardt’s solution = 1% each Ficoll 400, bovine serum albumin, and polyvinylpyrrolidone), 50 mM phosphate, pH 7.0, 1 mM EDTA, 250 μg/ml salmon sperm DNA, 500 μg/ml yeast tRNA. RNA probes were heated briefly at 85°C and cooled to 65°C before addition. Hybridization was carried out overnight at 55°C in heat-sealable bags. Filters were washed at 65°C in 0.1-0.3 × SSC, 1 mM EDTA, 0.1% SDS. Northern blot hybridizations with nick-translated DNA probes were conducted in 50% formamide, 4 × SSPE, 500 μg/ml salmon sperm DNA at 65°C. Filters were washed in 2 × SSC, 0.1% SDS at 50°C.

### DNA Probes

The mouse EGF receptor probes pME8.5 and pME8.0 have been described previously (20). The probe for the mouse β2-microglobulin gene (a gift from Michael Gilman) is a 0.9-kb HindIII/EcoRI subclone, containing most of exon II and part of the 5′-intron, in vector pSP65. The probe for the α-tubulin gene contains most of the genomic sequence encoding this gene (21). A subclone of the 5′-kb tubulin sequence in pBR322 was a gift from Phil Sharp. The fibronectin gene probe consists of 500 bases of the rat fibronectin cDNA sequence which encodes a portion of the C-terminal region of the molecule (22). This probe (clone λ-rfl1) had been subcloned into pBR325 and was a gift from Richard Hynes. The neo probe (neuc(t)/sp6400) contains a 420-bp BamHI fragment of the neo gene in vector pSP65. The sequence is conserved in the region of the gene encoding the putative extracellular domain and reportedly does not cross-hybridize with the EGF receptor sequence. neuc(t)/sp6400 was obtained from C. Bargmann and R. Weinberg (Whitehead Institute).

### Probe Labeling

RNA Probes—Prior to in vitro transcription, plasmid DNA was linearized with the appropriate restriction enzyme. Conditions for the synthesis of 32P-labeled RNA probes utilizing SP6 promoters were similar to those described by Melton et al. (23).

DNA Probes—DNA probes were labeled by nick translation with a kit from Bethesda Research Laboratories, according to the manufacturer's protocol. Typically, 0.1 μg DNA was labeled with 12.5 μCi of [γ-32P]dCTP (3000 Ci/mmol) for 1 h at 16°C. The reaction was stopped by the addition of EDTA to 10 mM, and the DNA probe was separated from free nucleotides on Blultips (Schleicher & Schuell) according to the manufacturer's instructions. The RNA was added to the sample prior to ethanol precipitation.

### Run-off Transcription Assays

#### Preparation of Nuclei—Nuclei were prepared from NRK cells by the Nonidet P-40 lysis procedure (24).

#### Labeling of Run-off Transcripts—Nuclear run-off transcripts were labeled by the method of Greenberg and Ziff (24) with several changes designed to increase specific activity and size of the transcripts. 100 μl of nuclei were thawed on ice and mixed with an equal volume of reaction mixture (10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 300 mM KCl, 500 μM of ATP, 500 μM CTP, 500 μM GTP, 3.5 μM unlabeled UTP, 100 ng/ml of [α-32P]UTP (3000 Ci/mmol, 1.5 μM). The nuclei were then incubated for 60 min at 30°C. 10 units of DNase I were added, and the samples were incubated at 37°C for 15-20 min. The nuclei were then lysed in 1 ml of GSCN, 100-200 μg of tRNA were added, and the samples were dispensed through a 26-gauge needle. After the addition of a second milliliter of GSCN, 50 μl of 1 M acetic acid, and 1.5 ml of ethanol, the samples were placed at -20°C overnight. The nuclear run-offs were precipitated by centrifugation at 8000 rpm in an SS 34 rotor for 20-25 min at -10°C. The pellets were resuspended in 3 ml of GSCN, and transcripts were isolated by overnight ultracentrifugation as described above. Transcript radioactivity was determined by scintillation counting. Run-off transcripts were normalized for equal amounts of radioactivity prior to hybridization.

#### Preparation of DNA Slot Blots—Linearized plasmid DNA was resuspended in TE, NaOH was added to 30 mM, and the mixture was incubated at 65°C for 30 min. After cooling on ice, RNA was added to 1 M, and the DNA was blotted onto nitrocellulose that had been prewet in 1 mM ammonium acetate using a Schleicher & Schuell slot blot apparatus. The slot blot was baked for 2 h in a vacuum oven at 80°C. Prior to hybridization, the blots were preincubated for at least 3 h in hybridization mixture (50% formamide, 5 × SSC, 2.5 × Denhardt’s solution, 100 μg/ml tRNA, 200 μg/ml calf thymus DNA). Blots were washed at 55°C in 0.2 × SSC, 0.1% SDS at 50°C. Run-off transcripts were hybridized with slot blots for 2 days in a shaking water bath at 50°C. Blots were washed at 55°C in 0.5 × SSC, 0.1% SDS, 1 mM EDTA. Ribonuclease treatment (10 μg/ml RNase A, 2 μg/ml RNase T1 in 2 × SSC for 30 min at 37°C) followed. After the blots were washed twice in 2 × SSC for 15 min at 37°C, they were analyzed by autoradiography and microdensitometry.

### RESULTS

The ability of retinoic acid to influence EGF receptor gene expression in NRK fibroblasts was first monitored by analysis of EGF receptor transcript levels. Since detection proved difficult with human EGF receptor cDNAs, we developed probes for the rodent EGF receptor gene (20). One genomic subclone was identified, corresponding to a region of the gene to the cytoplasmic kinase domain, that is highly specific for the EGF receptor gene and has a coding sequence that is strongly conserved among human, rat, and mouse genes. In Northern blot analyses, this probe (pME8.5) identifies 10- and 5.5-6-kb EGF receptor transcripts in human and murine cultured cells and a single 9-kb EGF receptor transcript in cultured rat cell lines (20). pME8.5 was used to prepare EGF receptor transcript levels in ribonuclease protection assays.
Retinoic acid induced a dose-related increase in EGF receptor mRNA levels in NRK cells (Fig. 1, lower). The elevation in transcript levels after 10 h of treatment paralleled the increase in EGF binding to NRK cells after 28 h of incubation with retinoic acid (Fig. 1, upper), suggesting that the increase in receptor transcript levels may fully account for the enhanced EGF binding. Maximal enhancements of mRNA levels of 3–5-fold were observed by 12 h after dosing with 1 μM retinoic acid (Fig. 2). The induction by retinoic acid was specific for the EGF receptor transcript. Retinoic acid had no effect in NRK cells on mRNA levels for fibronectin, α-tubulin, or β2-microglobulin (Fig. 2 and data not shown).

To test whether the retinoid-induced increase in transcript levels for the EGF receptor reflects regulation at the transcriptional level, run-off transcription assays were performed using nuclei prepared from treated and control NRK cells (24). Due to the low level of expression of the EGF receptor in NRK cells (approximately 10,000 receptors/cell), it was necessary to use a probe against a larger portion of the EGF receptor gene than is contained in the 0.5-kb pME0.5 clone. For these assays, we used pME8.0, an 8-kb genomic clone that encodes the kinase region of the mouse EGF receptor (20). Southern blot analysis indicated that this probe does not contain repeated sequences (data not shown). pME8.0 is weakly homologous in Northern and Southern blotting experiments with neu, a proto-oncongene that encodes tyrosine-specific protein kinase activity, and a putative growth factor receptor that is closely related to the EGF receptor gene in the kinase region (25). The specificity of binding of EGF receptor transcripts to pME8.0 in this assay had been previously demonstrated by the observation that TGF-β selectively induces run-off transcript binding to pME8.0 but not the neu-specific probe when assayed on the same filter (8).

Retinoic acid increased the rate of synthesis of EGF receptor gene transcripts. In nuclei prepared from NRK cells treated for 8 h with a dose of 1 μM retinoic acid, a 3–4-fold increase in transcription of the EGF receptor gene was observed (Fig. 3). Accurate quantitation was difficult due to the low rate of transcription of this gene in control nuclei. A similar treatment-related increase in the synthesis of nuclear transcripts hybridizing to specific (β2-microglobulin, tubulin) or nonspecific (pME0.5, pSP65 vector) probes was not observed (Fig. 3). Treatment with α-amanitin (0.5 μg/ml) abolished the synthesis of run-off transcripts hybridizing to the pME8.0 DNA probe in nuclei isolated from cells pretreated with retinoic acid (data not shown). These results clearly demonstrate that retinoic acid increases transcription of the EGF receptor gene in NRK cells.

Studies of mRNA stability provided further evidence for transcriptional regulation of the EGF receptor by retinoic acid. For this assay, NRK cells were incubated with 1 μM retinoic acid for 8 h, which corresponds to the time point when EGF receptor transcript levels are linearly increasing and are 3–4-fold above control levels. The cells were then treated with actinomycin D to inhibit transcription, and RNA was harvested after 2, 4, and 6 h. EGF receptor transcript levels were analyzed by the ribonuclease protection assay with 32P-labeled, PME0.5 RNA probes. First-order kinetics of decay were assumed in calculating transcript half-lives.

Retinoid treatment had no effect on the apparent half-life of the EGF receptor mRNA in NRK fibroblasts. Analysis of poly(A+) RNA from untreated NRK cells yielded a half-life

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**Fig. 1.** Effect of retinoic acid on EGF binding and EGF receptor mRNA levels in NRK cells. Upper, NRK cells were pretreated with retinoic acid for 28 h in 24-well dishes. Specific cell-associated radioactivity was determined after incubation with 125I-labeled EGF for 60 min at 37°C. Lower, NRK cells were incubated for 10 h in the presence of retinoic acid and harvested for RNA. EGF receptor transcript levels were determined by the ribonuclease protection assay in which 20 μg of total RNA were hybridized with 32P-labeled EGF for 60 min at 37°C. Retinoic acid increases the rate of synthesis of EGF receptor gene transcripts.

**Fig. 2.** Time course of effect of retinoic acid on transcript levels in NRK cells. mRNA was prepared from NRK cells after treatment with 1 μM retinoic acid for various times. EGF receptor (EGF-R) transcript levels in the samples were assayed by the ribonuclease protection assay with 32P-labeled pME0.5 RNA probes. α-Tubulin mRNA levels were determined by Northern blot analysis.

**Fig. 3.** Effect of retinoic acid on gene transcription in NRK cells. Following an 8-h pretreatment with 0 (control) or 1 μM (+RA) retinoic acid, nuclei were harvested, and nascent transcripts were elongated in vitro in the presence of [32P]UTP. After isolation, run-off transcripts were hybridized on nitrocellulose filters to DNA probes for tubulin (Tb), β2-microglobulin (β2Mg), EGF receptor (ER), or nonspecific hybridization by the vector (pME0.5). The autoradiograph was exposed for 4 days.
of ~1.3 h for the EGF receptor transcript (Fig. 4). When NRK cells were pretreated with retinoic acid and the total RNA was isolated after actinomycin D addition, the half-life of the EGF receptor mRNA was ~1.7 h. It should be noted that any indirect effect on EGF receptor mRNA decay that is sensitive to actinomycin D would not be detected in this experiment. In sum, these results suggest that retinoic acid does not increase EGF receptor mRNA levels by increasing message stability.

The ability of EGF, in combination with TGF-β, to enable NRK cells to grow in soft agar is potentiated by retinoic acid (12). This effect of retinoic acid has been attributed to its ability to enhance EGF binding. However, in other systems, EGF alone has been shown to increase EGF receptor mRNA and/or protein levels (2-4). Thus, to assess the role of EGF receptor gene expression in retinoic acid enhancement anchorage-independent growth, we analyzed the effect of EGF on EGF receptor expression in NRK cells.

EGF increased EGF receptor transcript levels in NRK cells approximately 2-fold at saturating doses (Fig. 5). The effect was specific since neither fibronectin nor tubulin transcript levels were significantly altered. The low level of induction of EGF receptor gene expression in NRK cells by EGF precludes analysis of the mechanism of this effect by the nuclear run-off transcription assay. The ability of retinoic acid to increase EGF receptor gene expression to a greater extent than EGF in NRK cells is consistent with the possible role of this effect in the mechanism of action of retinoic acid.

**DISCUSSION**

Our results indicate that a primary mode of positive regulation of EGF receptor gene expression of multiple growth modulatory agents is at the level of transcription. We have demonstrated that this is the effect of retinoic acid and of TGF-β in NRK cells by the nuclear run-off transcription assay. Like retinoic acid and TGF-β, EGF induces an increase in EGF receptor mRNA levels in NRK cells, although the maximal stimulation is only 2-fold. To determine the mechanism of action of this low level of induction would require a more sensitive assay, such as transfection with constructs containing the cloned EGF receptor promoter region linked to a reporter gene (e.g. luciferase or chloramphenicol acetyltransferase). Clark et al. (2), however, have reported that the induction of EGF receptor mRNA levels by EGF and phorbol esters occurs in the absence of an increase in transcription of the EGF receptor gene. Thus, the EGF receptor gene may be regulated via multiple mechanisms.

The ability of EGF to increase the synthesis of its receptor has been noted in a number of cultured cell lines (2-4). However, this effect of EGF is not obligatorily linked to mitogenicity. For example, EGF did not increase EGF receptor mRNA levels in mouse Swiss 3T3 fibroblasts, a line that responds mitogenically to EGF, although it did elevate myc mRNA levels at least 10-fold (26). Analysis of the cell types that are responsive to EGF induction of EGF receptor transcripts indicates that these are cells which show a strong mitogenic dependence upon EGF. Among these are epithelial cells which, unlike fibroblasts, lack receptors for the other mitogens, such as platelet-derived and fibroblast growth factors, that are present in serum. Thus, there may be a direct relationship between mitogenic dependence on EGF and induction of EGF receptor transcripts by growth activators.

The 5′-flanking region of the EGF receptor gene is unusual in that it lacks both a TATA box and a CAAT box, features which are characteristic elements of most eukaryotic promoters. Multiple transcription initiation sites have been observed and may be directed by repeated CCGCCC boxes thought to be binding sites for the transcription factor SP1 (27, 28). The points of regulation of EGF receptor promoter activity may thus be complex; and at present, it cannot be determined whether the multiple inducers of EGF receptor gene expression work through common or divergent regulatory pathways.

The intracellular receptor for retinoic acid is highly homologous to the thyroid and steroid hormone receptors which activate transcription through direct interaction with regulatory sequences in the 5′-flanking region of target genes (9, 10). Our evidence that retinoic acid increases EGF receptor gene transcription is consistent with this proposed mechanism of action of retinoic acid, although it is not yet known whether the retinoic acid receptor directly interacts with EGF receptor promoter or enhancer sequences.

The results presented here represent another mechanism by which the EGF receptor can be regulated. In previous studies, a number of biochemical regulatory pathways have been identified. For example, transient autophosphorylation of the receptor can lead to activation of the tyrosine kinase, and transient heterologous phosphorylation of the receptor by a number of growth modulators can lead to inactivation of the tyrosine kinase (reviewed in Ref. 29). EGF binding itself
induces an internalization and subsequent “down-modulation” of the receptor. All of the previous events occur rapidly, within 1 h of exposure to the regulatory agents. We have now demonstrated that the EGF receptor gene is also a target of receptor regulation. The kinetics of this type of transcriptional regulation are much longer than those of the biochemical regulatory mechanisms previously described and lead to a more stable change in the EGF receptor. In all cases that we have studied, the transcriptional change is a type of “up-modulation” leading to an enhancement in receptor number which could translate into an enhanced mitogenic response to EGF.

Acknowledgments—We would like to thank Drs. Ming-Chi Hung, Brent Cochran, and J. Victor Garcia for valuable discussions and assistance and Debbie Zeleznik for assistance with the manuscript.

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