Cloning of a novel EGFR-related peptide: a putative negative regulator of EGFR

YINGJIE YU,1 ARUN K. RISHI,1–3 JERROLD R. TURNER,4 DAYOU LIU,4 ERIC D. BLACK,4 JEFFREY A. MOSHIER,1,5 AND ADHIP P. N. MAJUMDAR1–3,5,6

2Veterans Affairs Medical Center, 3Karmanos Cancer Institute, and Departments of 1 Internal Medicine, 4 Pathology, and 6 Biochemistry and Molecular Biology, 5 Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, Michigan 48201

Received 28 September 2000; accepted in final form 14 November 2000

Yu, Yingjie, Arun K. Rishi, Jerrold R. Turner, Dayou Liu, Eric D. Black, Jeffrey A. Moshier, and Adhip P. N. Majumdar. Cloning of a novel EGFR-related peptide: a putative negative regulator of EGFR. Am J Physiol Cell Physiol 280: C1083–C1089, 2001.—Although epidermal growth factor receptor (EGFR) plays a key role in regulating cell proliferation, differentiation, and transformation in many tissues, little is known about the factor(s) that may modulate its function. We have isolated a cDNA clone from the rat gastroduodenal mucosa whose full length revealed 1,958 bp that contained 227 bp of 5′-untranslated region (UTR) and an open-reading frame encoding 479 amino acids, followed by 290 bp of 3′-UTR. It showed ∼85% nucleotide homology to the external domain of the rat EGFR. We refer to the product of the newly isolated cDNA as EGFR-related protein (ERRP). In Northern blot analysis with poly(A)+ RNA from different rat tissues, ERRP cDNA hybridized to several mRNA transcripts with the strongest reaction noted with a transcript of ∼2 kb. Maximal expression of the 2-kb mRNA transcript was observed in the small intestine, followed by colon, liver, gastric mucosa, and other tissues. Transfection of ERRP cDNA into a colon cancer cell line, HCT116, resulted in a marked reduction in proliferation in monolayer and colony formation in soft agar compared with the vector-transfected controls. In another colon cancer cell line, Caco-2, with a tetracycline-regulated promoter system, induction of ERRP expression in the absence of doxycycline was associated with a marked reduction in EGFR activation and proliferation. We conclude that the ERRP cDNA may represent a new member of the EGFR gene family and that ERRP plays a role in regulating cell proliferation by modulating the function of EGFR.

Cell proliferation; epidermal growth factor receptor; complementary deoxyribonucleic acid; epidermal growth factor receptor-related protein

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR), a 170-kDa transmembrane glycoprotein, and three other structurally related receptors (ErbB2/Neu, ErbB3, and ErbB4) are encoded by distinct genes (2, 8, 18, 19, 25). In addition, three splice variants of 1.8, 2.7, and 2.8 kb of the EGFR gene encoding the extracellular domain of the receptor have also been isolated (17, 21, 25). However, their functional roles remain unknown.

Accumulating evidence suggests that EGFR plays a key role in regulating proliferation, differentiation, and transformation of cells in many tissues (26). Overexpression of EGFR with increased tyrosine kinase activity has been associated with many malignancies, including cancers of the stomach and colon (1, 6, 7). In evaluating the regulation of gastrointestinal mucosal cell proliferation during aging, we have observed that the age-related rise in gastric mucosal proliferative activity is accompanied by a marked increase in tyrosine kinase activity and expression of EGFR and ErbB2 (4, 15, 23). These and several other conditions that stimulate proliferation and EGFR activation in the gastric mucosa (9, 11, 12, 16, 22) are also accompanied by parallel alterations in phosphorylation of a mucosal membrane protein with a molecular mass of 55 kDa (11, 12, 16), which we have referred to as pp55 (13). These observations led us to suggest that pp55 may play a role in modulating gastrointestinal mucosal cell proliferation (13).

To characterize pp55 and to study its functional properties, polyclonal antibodies raised against this protein (13) were used to screen a cDNA expression library generated from rat gastroduodenal mucosal mRNA. Interestingly, nucleotide sequence analysis of one of the candidate cDNA clones isolated from this screening showed a significant homology to the extracellular domain of the rat EGFR. It also revealed a substantial homology to the extracellular domain of the human EGFR (25). We referred to the product of this newly isolated cDNA as EGFR-related protein (ERRP). In this communication, we describe the properties of ERRP and propose a functional role as a negative regulator of EGFR.

METHODS

Isolation of RNA and Northern blot analysis. Total RNA was isolated from different rat tissues and a human colon carcinoma cell line, HCT116, using RNA-STAT solution (Tel...
ERRP: A NEW EGFR FAMILY MEMBER

Test, Friendswood, TX) according to the manufacturer’s instruction. Poly(A)+ RNA was derived from total RNA by oligo(dT) cellulose chromatography according to the instructions provided by GIBCO-BRL (Gaithersburg, MD). One microgram of poly(A)+ RNA was used for Northern blot analysis as described previously (14). The plasmid pBSK containing 1.6 kb of ERRP cDNA (see below) was linearized by digestion with PstI. The antisense RNA probe was then synthesized using 1 μg of linearized plasmid with T4 RNA polymerase with the use of a commercial kit (StrapAble RNA; Ambion, Austin, TX). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. GAPDH RNA probe was prepared by transcribing 1 μg of pTRI-GAPDH (rat; Ambion) with T3 RNA polymerase. The probes were labeled to a specific activity of 10^8 counts·min⁻¹·μg⁻¹ RNA with [α-32P]UTP followed by their hybridization with RNAs on the Northern blots according to the manufacturer’s instructions. After hybridization, the membranes were washed two times (15 min each time) with 2× saline-sodium-citrate (SSC) containing 0.1% SDS and one time with 0.5× SSC-0.1% SDS at 65–70°C. Membranes were exposed to X-omat film, and the intensity of the bands was analyzed by a PhosphorImager (Strom 860; Molecular Dynamics, Sunnyvale, CA).

Construction of cDNA expression library and screening. An unamplified cDNA expression library was constructed using the EcoRI cloning sites of the λgt11 vector system in Escherichia coli Y1090 strain according to the protocols recommended by Stratagene (La Jolla, CA). The library contained 3 × 10^10 plaque-forming U/ml. Nonrecombinant phage associated with the library was ~4%. Six duplicate isopropyl β-D-thiogalactopyranoside-treated nitrocellulose filters containing the imprint of ~50,000 plaques were washed with PBS-0.1% Tween 20 and left overnight at 4°C in a blocking buffer (PBS containing 7% nonfat dry milk, 0.5% BSA, and 0.1% Tween 20). Membranes were then washed at room temperature in PBS-0.1% Tween 20 and incubated for 2 h at room temperature followed by subsequent incubation for 2 h at room temperature with antibodies (1:5,000 final dilution) raised against a 55-kDa phosphotyrosine gastric mucosal membrane protein (13). Antibodies were preadsorbed six times with E. coli lysate protein according to the protocol provided by Stratagene. Bound pp55 antibodies were visualized by the enhanced chemiluminescence detection system (Amersham) according to the manufacturer’s instructions.

Sequence analysis. DNA from one of the positive plaques (referred to here as ERRP) was isolated and purified. Restriction analysis by EcoRI showed its size to be ~1.6 kb. This fragment was subcloned into the EcoRI site of pBluescript (KS+) vector and subsequently sequenced by the dideoxy chain termination method at the Core Facility of the Center for Molecular Medicine and Genetics, Wayne State University (Detroit, MI). The nucleotide sequence was compared with known sequences in GenBank using the NCBI BLAST search program.

Rapid amplification of cDNA ends. Because the candidate cDNA under study lacked the termination codon, rapid amplification of cDNA ends (RACE) was used to obtain the 3’-end in the following manner. Briefly, 1 μg poly(A)+ RNA from rat small intestinal mucosa was reverse transcribed into first-strand cDNA using oligo(dT) primer (5’-GACTCGAGTCGACATCGAGATTTTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT...
sion were plated in each well of 96-well tissue culture-treated multiwell plates (Corning) in standard growth media with 25 ng/ml doxycycline. Three days after plating, each well was washed three times with serum-free high-glucose DMEM without doxycycline. The final media in each well was serum-free media with or without doxycycline (25 ng/ml). Cells were harvested after 96 h for luciferase assay (Promega) and for proliferation by MTT assay. In a second set of experiments, ~1–3 × 10^7 cells/plate were cultured as indicated above in the absence or presence of doxycycline (25 ng/ml) for 96 h, subsequently harvested, and assayed for tyrosine kinase activity and the extent of EGFR phosphorylation as described below.

Tyrosine kinase activity of EGFR. The enzyme activity was determined in lysed cells as described previously (13, 23). Briefly, plated cells were lysed in lysis buffer (50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 1% Nonidet P-40, 2.5 mM Na_3VO_4, 25 μg/ml aprotinin, 25 μg/ml leupeptin, and 50 μg/ml soybean trypsin inhibitor). Aliquots of cell lysate containing 1 μg of protein were incubated overnight at 4°C with 1 μg polyclonal antibody to EGFR (Santa Cruz Biotechnology, Santa Cruz, CA). The immunocomplexes were precipitated with Sepharose-bound protein G and washed several times with kinase buffer (25 mM HEPES, pH 7.5, 5 mM MnCl_2, 2.5 mM MgCl_2, 0.1 M NaCl, 0.5 mM dithiothreitol, 0.5 mM Na_3VO_4, 10 mM p-nitrophenol phosphate, and 5 mM β-glycerol phosphate). The immunoprecipitates were resuspended in 30 μl of kinase reaction buffer (25 mM HEPES, pH 7.5, 10 mM MnCl_2, 2.5 mM MgCl_2, 150 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM Na_3VO_4, 10 mM p-nitrophenol phosphate, 5 mM β-glycerol phosphate, and 0.1 mM ATP) and assayed for tyrosine kinase activity by measuring 32P incorporation from [γ-32P]ATP into acid-denatured enolase (9). In addition, the extent of phosphorylation of EGFR was also monitored.

Statistical analysis. Where applicable, results were evaluated with Student's t-test for unpaired samples.

RESULTS

During the course of screening the cDNA expression library with pp55 antibodies, we obtained several candidate cDNA clones. However, nucleotide sequence analysis of one of the candidate cDNA fragment clones of 1,583 bp showed a substantial (~90%) homology to the extracellular domain of the rat EGFR. We referred to the product of this clone as ERRP. The 1,583-bp cDNA fragment possessed the "initiation" ATG codon but lacked the termination codon. In view of this, PCR-based RACE methodology was used to obtain overlapping clones with the carboxy terminus sequences. This resulted in a translation termination codon followed by ~290 nucleotides of 3'-UTR containing a polyadenylation signal sequence (5'AAUAAA-3') and a short stretch of poly(A)' tail. The full-length cDNA thus obtained consisted of 1,958 base pairs that included a 227-bp 5'-UTR, a putative open reading frame (ORF) of 1,437 nucleotides encoding for 479 amino acids, and a 290-nucleotide-long 3'-UTR. The nucleotide sequence of the ORF showed ~85% homology to the rat EGFR (GenBank accession no. = AF187818; Fig. 1A). It also revealed a little over 80% homology to the external domain of the human EGFR. However, the first 24 amino acids of the ORF were similar but not identical to those found in the signal peptide of human and rat EGFR, and the first 16 nucleotides of the 5'-UTR of ERRP revealed no homology with any other known sequences (Fig. 1B). Additionally, a region of the 3'-end (from nucleotide 1580 to 1661) encoding 27 amino acids also showed no homology with any known sequence in the current data bases.

To evaluate the possible expression of putative ERRP, Northern-blot analysis was performed with poly(A)^+ RNA from different tissues of the gastrointestinal tract and from the liver, brain, heart, and testes from rats. Expression of ERRP mRNA varied considerably among different tissues (Fig. 2). ERRP cDNA hybridized strongly to an mRNA transcript of ~2 kb, with maximal expression noted in the small intestine, followed by colon, liver, gastric mucosa, and other tissues (Fig. 2). No hybridization of ERRP cDNA to the ~2.0-kb mRNA transcript was observed in testes, brain, and heart (Fig. 2). In addition to the ~2.0-kb mRNA transcript, ERRP cDNA also reacted strongly with the 2.8-kb transcript in the liver and with 0.6-, 2.8-, and 5.0-kb mRNAs in certain tissues, most notably in the colon (Fig. 2). The transcript size of 5.0 kb reacting with the ERRP probe probably represents the EGFR mRNA. No expression of ERRP mRNA was observed in the heart (Fig. 2).

To determine the putative functional properties of ERRP, a 1.6-kb fragment ERRP cDNA was stably transfected into HCT116 cells, a colon cancer cell line. Two ERRP-positive clones, as evidenced by RT-PCR analysis, with primers generated from the 1.6-kb fragment of the full-length 1.95-kb ERRP were selected. ERRP expression was found to be slightly higher in clone 1 than clone 2 (Fig. 3). No ERRP mRNA was detected in control cells transfected with the vector.
alone (Fig. 3). GAPDH mRNA levels were found to be very similar among the different clones (Fig. 3). These clones were subsequently used to determine whether transfection of ERRP might affect proliferation of HCT116 cells. When these clones were maintained in complete medium containing either 10% FBS or 0.1% FBS (serum starved), we observed a marked reduction (50–70%) in proliferation of ERRP-transfected cells compared with the controls (Fig. 4A). However, when these clones were maintained in 10% FBS, inhibition of proliferation of the ERRP-transfected clones became evident only after 4 days (Fig. 4A). On the other hand, under serum-starved conditions, inhibition of proliferation of ERRP-transfected cells could be observed as early as the 2nd day in culture (Fig. 4B). The magnitude of inhibition was also exacerbated when the ERRP-transfected clones were serum starved (0.1% FBS; Fig. 4B). This could be due to the lack of growth factors, including the EGF family of peptides. To demonstrate that the inhibition of HCT116 cell proliferation by ERRP expression was not due to a “position effect” of the inserted DNA, we assayed three other distinct ERRP-transfected HCT116 cell subclones. Each of these subclones exhibited decreased proliferation relative to controls (data not shown). Moreover, we have recently observed that transfection of ERRP cDNA into a prostate cancer cell line, PC-3, also inhibits proliferation (data not shown), implicating an antiproliferative role for ERRP in different epithelial cancer cells.

In the next set of experiments, transforming growth factor (TGF)-α-induced proliferation of ERRP- and vec-

---

**Fig. 2.** Representative Northern blot analysis showing the mRNA transcript size of ERRP in different tissues from rats. Each lane contains 2 μg poly(A)⁺ RNA. The membrane was also probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Histogram shows changes in relative concentration of ERRP mRNA, expressed as a ratio of ERRP to GAPDH. Experiment was repeated at least 3 times. Int, intestine.

**Fig. 3.** RT-PCR reaction showing levels of ERRP and GAPDH in control (vector transfected) and ERRP cDNA-transfected (clones 1 and 2) HCT116 cells. RT-PCR was performed with primers for ERRP and GAPDH simultaneously (lanes 1–3) or with primers for ERRP (lanes 4–6) or GAPDH (lanes 7–9). Lanes 1–3, clones 2 and 1 and controls, respectively; lanes 4–6, clones 2 and 1 and controls, respectively; lanes 7–9, clones 2 and 1 and controls, respectively. L, DNA ladder.

**Fig. 4.** Effect of ERRP cDNA transfection on proliferation of HCT116 cells. Control (vector transfected) and ERRP cDNA-transfected cells were maintained in DMEM-10% FBS (A) or DMEM-0.1% FBS (serum starved; B). Each value represents mean ± SE for 6 observations. OD₅₆₄, optical density at 564 nm.
tor-transfected HCT cells was examined. In this investigation, serum-starved (0.1% FBS) cells from clones 1 and 2 and from the vector-transfected clone were maintained for 24 h in the absence (basal) or presence of increasing concentrations of TGF-α. As shown in Table 1, TGF-α at a dose of either 0.1 and 1 nM, but not 0.01 nM, significantly stimulated proliferation in control cells but had no effect on ERRP-transfected cells.

Table 1. Effect of TGF-α on proliferation of HCT116 cells transfected with ERRP cDNA or the vector

<table>
<thead>
<tr>
<th>HCT116 sublines</th>
<th>TGF-α, nM</th>
<th>0</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector transfected (control)</td>
<td>0.43 ± 0.022</td>
<td>0.46 ± 0.017</td>
<td>0.51 ± 0.01*</td>
<td>0.57 ± 0.015†</td>
<td></td>
</tr>
<tr>
<td>ERRP transfected</td>
<td>Clone 1</td>
<td>0.42 ± 0.006</td>
<td>0.40 ± 0.023</td>
<td>0.42 ± 0.025</td>
<td>0.37 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>Clone 2</td>
<td>0.43 ± 0.008</td>
<td>0.43 ± 0.005</td>
<td>0.44 ± 0.025</td>
<td>0.46 ± 0.021</td>
</tr>
</tbody>
</table>

Results are means ± of 5–6 observations. 7,000 cells/well were seeded in DMEM-10% FBS followed by serum starvation for 72 h. Cells were then incubated with increasing doses of transforming growth factor (TGF)-α for 24 h followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *P < 0.05 and †P < 0.01 compared with cells incubated in the absence of TGF-α.

To determine whether overexpression of ERRP in HCT116 cells will also affect their transformed properties, ERRP-transfected HCT116 cells (clones 1 and 2) and their control counterpart were grown in soft agar for 14 days. As shown in Fig. 5, the number of colonies formed by clones 1 and 2 were 65 and 40% lower, respectively, compared with the control clone. The overall size of the colonies formed from ERRP-transfected HCT116 and PC-3 subclones was considerably smaller than that formed from the corresponding vector-transfected controls (data not shown).

Although the regulatory mechanisms for the ERRP-induced inhibition of cell proliferation remain to be fully elucidated, we hypothesize that ERRP may inhibit proliferation by attenuating EGFR activation. To test this hypothesis, we generated sublines from another colon cancer cell line (Caco-2) in which ERRP expression was regulated by a tetracycline-inducible promoter system. We isolated two clones with regulated expression of ERRP, which revealed 25- to 30-fold upregulation of ERRP in the absence of tetracycline (doxycycline) as observed by increased ERRP mRNA levels by RT-PCR (data not shown). One of the clones was assayed for ERRP-dependent growth and EGFR tyrosine kinase activity. Results revealed that the absence of doxycycline caused an ~300-fold increase in luciferase activity (Fig. 6A). In the absence of doxycycline, ERRP expression was also augmented, resulting in inhibition of proliferation and tyrosine kinase activity and the extent of phosphorylation of EGFR by 40–75% compared with the corresponding levels in clones grown in the presence of the antibiotic (Fig. 6, B and C). The discrepancy between induction and luciferase activity and inhibition of cell proliferation could partly be the result of differences in stability of luciferase and induction of ERRP expression.
erase and EGFR activity. The precise reason for this discrepancy still remains to be determined. Western blot revealed no significant differences in EGFR between doxycycline-treated and untreated cells, indicating the inhibition of EGFR activation could not be attributed to changes in EGFR levels (Fig. 6B).

**DISCUSSION**

EGFR, a 170-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity that binds the EGF family of peptides, plays an important role in controlling cell proliferation, differentiation, and transformation (25, 26). The EGFR possesses three functional domains that include extracellular, transmembrane, and cytoplasmic. Ligand binding to the extracellular domain of EGFR leads to dimerization and activation of the receptor’s intrinsic tyrosine kinase activity, located in the cytoplasmic domain, triggering a complex array of enzymatic and biological events leading to cell proliferation and differentiation (25, 26).

Overexpression of EGFR has been shown to be associated with many malignancies, including cancers of the stomach and colon (1). Evidence is accumulating that shows that malignant and certain normal cells also produce other form(s) of EGFR. For example, A 431 human epidermoid carcinoma cells have been shown to produce a truncated EGFR that encodes a 2.8-kb mRNA transcript (25) that is thought to be the result of gene rearrangement in chromosome 7 (25). Likewise, the normal rat liver produces a 2.7-kb mRNA transcript whose 5′, but not 3′, sequences show 100% homology with the external domain of the full-length rat EGFR (17). This and other relevant observations suggest that this truncated form of EGFR mRNA, whose protein product is also secreted from the cell, is generated from alternative splicing of the primary EGFR transcript (17). A shorter 1.8-kb alternative transcript from the human EGFR, which also produces a secreted product, has been isolated from a human placental cDNA library (21).

However, as opposed to the above-described forms of EGFR which were derived from a single gene, the ERRP cDNA that we isolated from the rat gastroduodenal mucosal cDNA expression library and which corresponded to a 2-kb mRNA transcript does not appear to be the product of the primary EGFR transcript. The basis for this inference comes from the observation that nucleotide and amino acid sequences between this and other forms of EGFR show significant differences. Nucleotide and amino acid sequences of ERRP show 84 and 89% homology with those from the external domain of the rat EGFR. Furthermore, although the 5′-UTR of ERRP showed significant homology with the corresponding region of the rat EGFR (10), the ERRP transcription start site appears to be unique. Rat EGFR mRNA has been demonstrated to contain multiple start sites [R1–R6 (10)]. On the other hand, the ERRP 5′-UTR sequences upstream of the corresponding R4 start site of rat EGFR are divergent, suggesting a possible unique transcription start site for the ERRP gene. In addition, the putative signal peptide derived from the ERRP cDNA shows amino acid alterations compared with the signal peptide of rat EGFR. Moreover, the putative ORF of the ERRP cDNA contains a stretch of 27 amino acids, referred to as the “U” region located at the carboxy terminus (see Fig. 1), which showed no homology with the rat truncated EGFR mRNA. Although additional experiments are undoubtedly necessary to fully characterize ERRP, on the basis of the observations made thus far, we suggest that ERRP is a member of the EGFR family of peptides but not a product of the EGFR gene. The fact that ERRP is primarily expressed in the gastrointestinal tract and liver and not in other tissues such as the heart, brain, and testes suggests that it functional role may be restricted to certain tissues. The presence of high levels of ERRP mRNA in the gastrointestinal tract, where EGFR plays a critical role in regulating cell proliferation (1, 20), suggests that ERRP may regulate EGFR in the gastrointestinal tract.

Although the functional properties of ERRP remain to be fully elucidated, our observation that overexpression of ERRP in HCT116 cells lowers their ability to grow in monolayer suggests a role for this protein in modulating cell proliferation. This postulation is further strengthened by the observation that induction of ERRP expression in Caco-2 cells (where ERRP is regulated by doxycycline) is associated with a marked reduction in proliferation. Additional support comes from the observation that the ERRP-transfected HCT116 cells also show a decreased ability to grow in soft agar. Because the latter is used to assess transformed properties of cells, our observation that overexpression of ERRP in HCT116 cells attenuates their ability to form colonies in soft agar further suggests a plausible antitumorigenic property of ERRP. The mechanism(s) by which ERRP inhibits cellular growth is unknown. However, our observation that, in Caco-2 cells with a tetracycline-inducible promoter system, induction of ERRP expression in the absence of doxycycline is associated with inhibition of proliferation as well as tyrosine kinase activity and phosphorylation of EGFR suggests that ERRP may exert its antiproliferative effect by inhibiting EGFR function. Interestingly, a human EGFR cDNA, generated by insertion of a synthetic linker resulting in expression of truncated EGFR containing the extracellular and transmembrane domains, when transfected into pancreatic cancer cells (PANC-1), caused a marked inhibition of EGFR and TGF-α-induced EGFR tyrosine phosphorylation (27). However, the mechanism(s) by which ERRP inhibits EGFR activation remains to be elucidated.

This work was supported National Institutes of Health Grants AG-14543 (A. P. N. Majumdar) and DK-02503 and DK-56121 (J. R. Turner) and by the Department of Veterans Affairs (A. P. N. Majumdar).

**REFERENCES**

1. Barnard JA, Beauchamp J, Rissell WE, Dubois R, and Coffey R. Epidermal growth factor-related peptides and their


