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A variant epidermal growth factor receptor exhibits altered type α transforming growth factor binding and transmembrane signaling

(protooncogenes)

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ABSTRACT **Epidermal growth factor (EGF) and type α transforming growth factor (TGF-α) bind to a specific region in subdomain III of the extracellular portion of the EGF receptor (EGFR). Binding leads to receptor dimerization, auto- and transphosphorylation on intracellular tyrosine residues, and activation of signal transduction pathways. We compared the binding and biological actions of EGF and TGF-α in Chinese hamster ovary (CHO) cells expressing either wild-type human EGFR (HER497R) or a variant EGFR that has an arginine-to-lysine substitution in the extracellular domain at codon 497 (HER497K) within subdomain IV of EGFR. Both receptors exhibited two orders of binding sites with radioiodinated EGF (^{125}I-EGF). Similar results were obtained with ^{125}I-TGF-α in cells expressing HER497R. In contrast, only one order of low-affinity binding sites was seen with ^{125}I-TGF-α in the case of HER497K. Although EGF and TGF-α enhanced tyrosine phosphorylation of both receptors, CHO cells expressing HER497K exhibited an attenuated growth response to EGF and TGF-α and a reduced induction of the protooncogenes *FOS*, *JUN*, and *MYC*. Moreover, high concentrations of TGF-α (5 nM) inhibited growth in these cells but not in cells expressing HER497R. These findings indicate that a region in subdomain IV of EGFR regulates signal transduction across the cell membrane and selectively modulates the binding characteristics of TGF-α.**

The family of transmembrane tyrosine kinase receptors constitutes an important group of regulatory proteins, which include the insulin receptor and the epidermal growth factor receptor (EGFR). Ligand specificity is conferred by the presence of unique sequences in the extracellular domain of each receptor. Often, more than one ligand can bind to the same receptor. Thus, the insulin receptor binds insulin and, with a lesser affinity, insulin-like growth factors I and II (1). EGFR binds EGF, type α transforming growth factor (TGF-α), amphiregulin, betacellulin, and heparin-binding EGF-like growth factor (2, 3). Ligand binding in the extracellular domain leads to receptor autophosphorylation on tyrosine residues located within the intracellular domain and initiation of a cascade of biochemical reactions that mediate the biological actions of many hormones and growth factors (1–3). In the absence of ligand, the extracellular domain acts through unknown mechanisms to prevent constitutive and unregulated activation of the intrinsic tyrosine kinase activity of the intracellular domain (4, 5).

The extracellular domain of transmembrane tyrosine kinase receptors often contains different structural subdomains. In the case of EGFR, there are four contiguous regions consisting of subdomain I at the N terminus and the cysteine-rich subdomains II and IV flanking subdomain III (6). Cross-linking studies have identified a 47-amino acid sequence (residues 321–367) within subdomain III as the EGF/TGF-α binding region (6, 7). The specific functions of the other extracellular subdomains are not known.

Recently, a variant EGFR has been identified that has an arginine-to-lysine substitution at codon 497 (8). This variant receptor was found in normal human lymphocytes and a number of cultured human cancer cell lines but not in A431 cells (8). Inasmuch as the potential role of this region of EGFR with respect to modulation of cell proliferation and gene activation is not known, in the present study we sought to characterize the biological properties of HER497K. Accordingly, we expressed the wild-type human EGFR (HER497R) and HER497K in Chinese hamster ovary (CHO) cells devoid of endogenous EGFR and determined whether there are differences between the two receptors with respect to ligand binding, cell growth, and induction of the immediate early response genes *FOS*, *JUN*, and *MYC*.

MATERIALS AND METHODS

Materials. The following materials were purchased: receptor grade murine EGF from Collaborative Research; GF/F filters from Whatman; PY-20 anti-phosphotyrosine antibodies from ICN; ^{125}I, [α-^{32}P]dCTP, ECL blotting kit, and Hyperfilm-ECL from Amersham; Iodo-Gen from Pierce; pGEM-7Zf(+) plasmid from Promega; pSVK 3 expression vector from Pharmacia; human *MYC*, *FOS*, and *JUN* cDNAs from the American Type Culture Collection; GeneScreen membranes from New England Nuclear; Qiagen minicolumns; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma; and Ecoscint scintillation fluid from National Diagnostics. Recombinant human TGF-α, 13A9 monoclonal anti-EGFR antibodies (9), and CHO cells were gifts from M. Winkler and B. Fendley (Genentech, South San Francisco).

Generation of Expression Vectors. Two overlapping oligonucleotides were synthesized (8) and used to generate a linker that contained the following restriction sites: *Sph* I/*Hin*dIII/*Sac* I/*Eco*RI/*Bgl* II/*Sma* I/*Sal* I/*Bst*XI. The linker was subcloned into the cloning vector pGEM-7Zf at the *Sph* I/*Bst*XI sites, generating the vector pGEM7.2Zf. Four *Eco*RI fragments (HERA, HERB, HERC, and HERD) encompassing the entire EGFR coding region were isolated from a λ library derived from T_3M_4 human pancreatic cancer cells, with HERB containing the nucleotide base change (8). The wild-type EGFR expression plasmid fragment was prepared by substituting HERB with fragment HERG, originally cloned from A431 cells (2, 25). The four EGFR fragments

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Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; TGF-α, type α transforming growth factor; CHO, Chinese hamster ovary; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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were reassembled as follows: the 314-bp *Sac* I/*Eco*RI fragment of HERA, the 1841-bp *Eco*RI HERB (or HERG) fragment, and the 637-bp *Eco*RI/*Bgl* II fragment of HERC were sequentially subcloned into pGEM-7.2Zf, generating plasmid HER7.2ABC. The 771-bp *Eco*RI HERC fragment and the 884-bp *Eco*RI/*Sca* I fragment of HERD were sequentially subcloned into pGEM-7.2Zf, generating plasmid HER7.2CD. A 1025-bp *Bgl* II/*Sal* I fragment from HER7.2CD was then subcloned into HER7.2ABC, yielding a full-length EGFR cDNA. The two 3.8-kb *Hin*dIII/*Sal* I EGFR cDNAs were then subcloned into the pSVK 3 expression vector under the control of the simian virus 40 early promoter, yielding pHER497K (variant EGFR) and pHER497R (wild-type EGFR). Authenticity of the constructs was confirmed by sequencing. Transfection of pHER497K, pHER497R, and pSV2-*dhfr* DNA into CHO cells deficient in synthesis of dihydrofolate reductase was carried out by the calcium phosphate precipitation technique (10). After reaching confluency, cells were plated at a 1:10 dilution in selection medium [Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (1:1) lacking hypoxanthine, glycine, and thymidine] supplemented with 5% dialyzed fetal bovine serum (FBS). After 2 weeks, single clones of cells were isolated, and each individual clone was plated separately. After clonal expansion, cells from each individual clone were tested for EGFR expression by Northern blot analysis and ^{125}I-labeled EGF (^{125}I-EGF) binding. The selected clones were shown to express only the variant HER497K or wild-type HER497R by subjecting RNA samples to reverse transcriptase PCR amplification followed by asymmetric PCR and sequencing (8).

Binding Experiments. Biologically active ^{125}I-EGF (40–50 μCi/μg; 1 Ci = 37 GBq) and ^{125}I-TGF-α (50–70 μCi/μg) were iodinated with Iodo-Gen and chloramine T, respectively (11). Binding was performed at 4°C for 5 hr on cells in monolayer culture (70–80% confluent) with increasing concentrations of labeled ligand and DMEM containing 0.1% bovine serum albumin (BSA) and 20 mM Hepes (11). Incubations were stopped by washing cells with phosphate-buffered saline containing 0.1% BSA. Nonspecific binding, determined in the presence of a 1000-fold excess of unlabeled EGF, did not exceed 10% of total binding. Equilibrium binding data were analyzed with the LIGAND program (12).

Cell Growth Assay. Cell proliferation was assayed by measuring the metabolism of the tetrazolium salt MTT and by monitoring [^{3}H]thymidine incorporation into DNA (13). The MTT assay measures the cell's ability to metabolize MTT, which correlates well with the rate of cellular proliferation (13). For the MTT experiments, cells were plated at a density of 8×10^3 cells per well in 96-well plates and grown overnight in 0.2 ml of DMEM/Ham's F-12 medium (1:1) containing 5% FBS. After replacing the medium with 0.2 ml of the same medium containing 0.5% FBS, cells were incubated for 72 hr at 37°C in the absence or presence of ligands, prior to the addition of MTT (5 mg/ml) for 4 hr (13). Reactions were stopped with acidified isopropanol (0.04 M), and the absorbance of the supernatants was measured at 570 nm with an ELISA plate reader (Molecular Devices). Absorbance readings were converted to the percentage of control growth, which was determined in the absence of growth factors and defined as 100% (13). For the [^{3}H]thymidine incorporation experiments, cells were plated at a density of 3×10^4 cells per well in 12-well plates and incubated for 48 hr in 1.5 ml of DMEM/Ham's F-12 medium (1:1) containing 5% FBS. Cells were then incubated for 40 hr in the absence or presence of ligand with serum-free medium containing 5 μg of insulin per ml, 5 μg of transferrin per ml, 5 ng of selenium per ml, and 0.1% BSA before adding [^{3}H]thymidine (1 μCi/ml) for 1 hr. Cells were collected by rapid filtration on GF/F filters and washed sequentially with 10% trichloroacetic acid, 70% ethanol, and 100% ethanol. Radioactivity was measured in a liquid scintillation counter using Ecoscint as the scintillant. Data were then expressed as percentage change from control values.

EGFR Phosphorylation. Cells were incubated for the specified times at 37°C in binding medium in the absence or presence of each ligand and then solubilized in buffer containing 50 mM Tris·HCl (pH 7.7), 150 mM NaCl, 5 mM EDTA, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 0.1 mM Na_3VO_4 (14). Cell homogenates or immunoprecipitated EGFR (using the 13A9 anti-EGFR antibodies) were subjected to SDS/7.5% PAGE and transferred to nitrocellulose membranes (14). Membranes were incubated with PY-20 anti-phosphotyrosine antibodies (1 μg/ml) for 1 hr, washed, and subjected to chemiluminescence Western blotting with the ECL blotting kit and Hyperfilm-ECL.

Northern Blot Analysis. Total RNA was isolated by the acid guanidium thiocyanate extraction method (15), size-fractionated, and transferred onto GeneScreen membranes (16). Filters were hybridized under high-stringency conditions using [α-^{32}P]dCTP-labeled cDNAs (16). The intensity of the radiographic bands was quantified by laser densitometry.

Statistical Analysis. Statistical analysis of the experimental results was obtained by one-tailed Student's *t* test using the STATVIEW computer program, with $P < 0.05$ taken as significant.

RESULTS

Characterization of Ligand Binding. EGF and TGF-α binding and dissociation are pH dependent (14). Therefore, to determine EGFR affinity and capacity, equilibrium binding studies (4°C) were carried out at pH 7.4 (Fig. 1) and pH 8.0 (Table 1). Computer-based analysis of ^{125}I-EGF binding data (12) demonstrated the presence of two orders of binding sites at the two pH levels in CHO cells expressing either wild-type HER497R or variant HER497K. At pH 7.4, there were approximately 90,000 and 116,000 low-affinity receptors in cells expressing HER497R and HER497K, respectively (Table 1). The number of high-affinity binding sites (3700 and 4800) was also similar in both cell types (Table 1). At pH 8.0, there was a decrease in the number of binding sites for EGF with both receptors and a concomitant increase in binding affinity (Table 1). ^{125}I-TGF-α also exhibited two orders of binding sites in cells expressing HER497R. As in the case of EGF, there was a decrease in the number of TGF-α binding

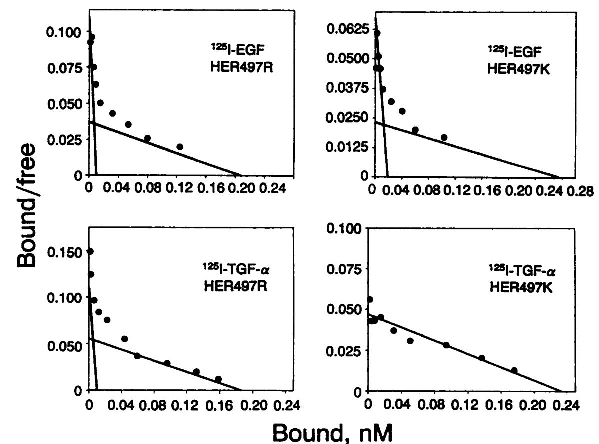

FIG. 1. Scatchard analysis. CHO cells expressing the indicated EGFR were incubated for 5 hr (4°C) in six-well plates with binding medium (pH 7.4) containing increasing concentrations (0.1–80 ng/ml) of either ^{125}I-EGF or ^{125}I-TGF-α. Data are representative of two experiments.

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Table 1. Binding characteristics of ^{125}I-EGF and ^{125}I-TGF-α

		Receptor, no. per cell		K_d, nM	
pH	Ligand	HER497R	HER497K	HER497R	HER497K
7.4	EGF	89,900	116,200	5.73	13.6
		3,700	4,800	0.1	0.28
7.4	TGF-α	123,300	158,100	4.57	5.62
		8,200		0.16	
8.0	EGF	47,300	83,800	3.39	8.49
		1,500	2,900	0.05	0.17
8.0	TGF-α	61,600	91,400	2.26	3.75
		1,100		0.02	

HER497R- and HER497K-expressing cells were incubated for 5 hr (4°C) at the indicated pH in six-well plates with increasing concentrations of either ^{125}I-EGF or ^{125}I-TGF-α (0.1–80 ng/ml). Data were analyzed with the LIGAND program as described and are means of two separate experiments with each ligand.

sites at pH 8.0 in conjunction with an increase in binding affinity (Table 1). However, irrespective of the pH, only a single order of low-affinity binding sites was observed with ^{125}I-TGF-α in cells expressing HER497K (Table 1).

To determine the effect of pH on ligand dissociation, cells were initially incubated for 5 hr at 4°C (pH 7.4) with either ^{125}I-TGF-α or ^{125}I-EGF, washed at 4°C, and incubated for 2 hr at various pH levels (4°C) in fresh medium devoid of radioactivity (Fig. 2). At pH 4, the majority of ^{125}I-EGF was released from the cells. Raising the pH caused a progressive decrease in the percentage of dissociated radioactivity, with 50% dissociation occurring at pH 4.8 and 5.4 with HER497K and HER497R, respectively. The least dissociation occurred at pH 6.5–7.0, and the amount of ^{125}I-EGF dissociated was similar with HER497R and HER497K. In contrast, with both receptors, the majority of ^{125}I-TGF-α was still dissociated from the cells at pH 5, and 50% dissociation occurred at pH ≈7.0. However, at pH 8 and 8.5, ^{125}I-TGF-α dissociated more readily from HER497K than from HER497R.

Growth Stimulation by EGF and TGF-α. As determined by the MTT dye reduction assay, EGF and TGF-α modulated the growth of EGFR-expressing CHO cells. In the case of HER497R, maximal stimulation of 40% occurred at concentrations of 0.1–0.8 nM with either growth factor (Fig. 3 *A* and *C*). Higher ligand concentrations resulted in a slight decrease in growth stimulation. In the case of HER497K, there was only a minimal increase in cell growth with EGF (0.1 nM) and none with TGF-α (Fig. 3 *A* and *C*). Furthermore, at high concentrations (2–5 nM), TGF-α inhibited cell proliferation (Fig. 3*C*). When [^{3}H]thymidine incorporation was assayed, maximal stimulation of 45–90% occurred at concentrations of 0.2–2 nM EGF and TGF-α with HER497R (Fig. 3*B*). In contrast, with HER497K, low concentrations of EGF increased [^{3}H]thymidine incorporation by 45%, whereas TGF-α was without effect. Conversely, high concentrations of TGF-α (1.5–5 nM) markedly inhibited [^{3}H]thymidine incorporation, and EGF was without effect (Fig. 3*D*).

Effects of EGF and TGF-α on EGFR Phosphorylation. To determine whether EGF and TGF-α induce tyrosine phosphorylation of HER497K, immunoblotting experiments were carried out with anti-phosphotyrosine antibodies. Both EGF and TGF-α (34 nM) increased the phosphotyrosine content of HER497K (Fig. 4*A*). Maximal effects were seen 2–5 min after addition of either EGF or TGF-α (Fig. 4*A*). To confirm that the phosphorylated band represented HER497K and that HER497R was also phosphorylated, EGFR was immunoprecipitated from CHO cells expressing HER497K and HER497R using an anti-EGFR antibody (13A9), followed by immunoblotting with the anti-phosphotyrosine antibodies.

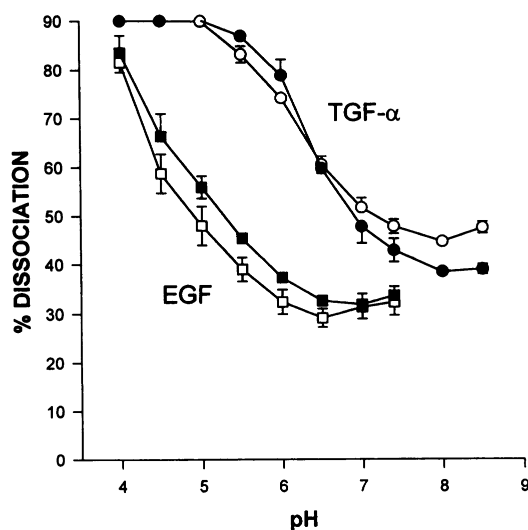

FIG. 2. Effect of pH on ligand dissociation. HER497R-expressing (solid symbols) and HER497K-expressing (open symbols) cells were incubated for 5 hr (4°C) in six-well plates with binding medium (pH 7.4) containing 200,000 cpm of ^{125}I-EGF (squares) or ^{125}I-TGF-α (circles) and then washed and incubated for 2 hr at 4°C in fresh medium at the indicated pH. Radioactivity dissociated from the cells during the 2-hr incubation was expressed as a percentage of the radioactivity that bound to the cells during the initial 5-hr incubation. Data are means ± SE of duplicate determinations from three experiments.

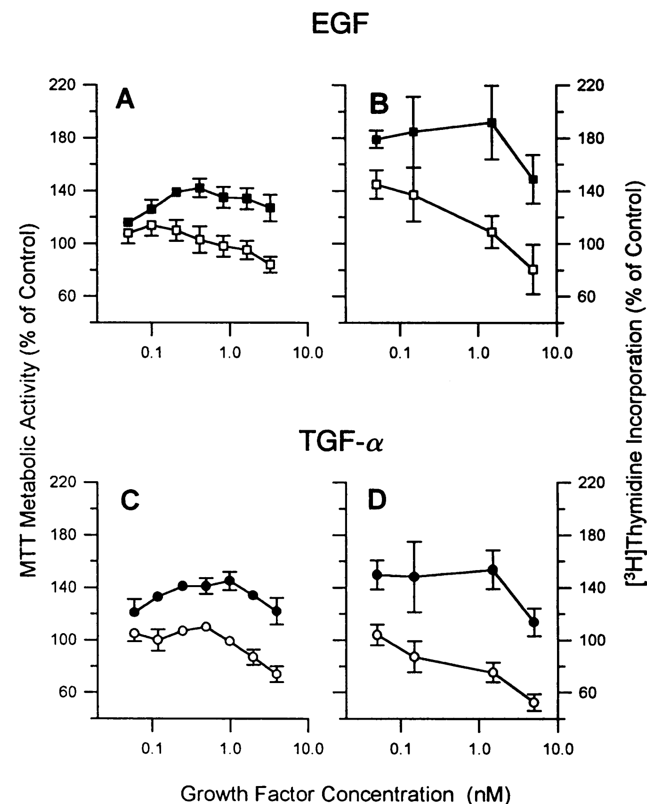

FIG. 3. Effects of EGF and TGF-α on cell growth. HER497R-expressing (solid symbols) and HER497K-expressing (open symbols) cells were incubated for 72 hr in the absence or presence of the indicated concentrations of EGF and TGF-α. Cell proliferation was determined by the MTT dye reduction assay (*A* and *C*) and by monitoring [^{3}H]thymidine incorporation (*B* and *D*). Control values, determined in the absence of growth factor, were defined as 0. Data are expressed as percentage change from control values and are means ± SE of quintuplicate determinations from three experiments (MTT) or triplicate determinations from three experiments ([^{3}H]thymidine incorporation).

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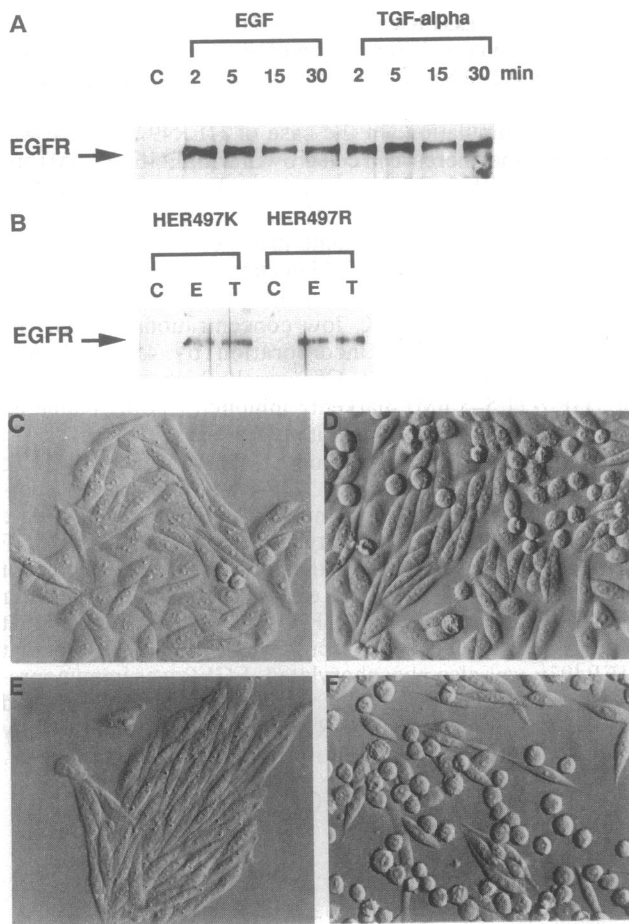

FIG. 4. Effects of EGF and TGF-α on phosphotyrosine content and cell morphology. (*A*) HER497K-expressing cells were incubated at 37°C with 34 nM EGF or TGF-α for the indicated times. Samples were lysed, subjected to SDS/PAGE, and transferred to membranes. The membranes were incubated with the PY-20 anti-phosphotyrosine antibodies and treated with ECL Western blotting reagents. (*B*) HER497R- and HER497K-expressing cells were incubated for 2 min at 37°C with 3.4 nM EGF (lanes E) or TGF-α (lanes T). HER497R and HER497K were then immunoprecipitated, and the immunoprecipitates were subjected to immunoblotting with PY-20 antibodies as described above. Lanes C, control. (*C–F*) Cell morphology. HER497R- (*C* and *D*) and HER497K- (*E* and *F*) expressing cells were grown in six-well plates in medium containing 0.5% FBS in the absence (*C* and *E*) or presence (*D* and *F*) of 3.4 nM EGF. (×320.)

Both EGF and TGF-α (3.4 nM) enhanced tyrosine phosphorylation of HER497K and HER497R (Fig. 4*B*), even at concentrations as low as 250 pM (data not shown).

Effects of EGF and TGF-α on *MYC*, *FOS*, and *JUN* mRNA Levels and Cell Morphology. In both HER497K- and HER497R-expressing cells, EGF (1.7 nM) caused a time-dependent increase in the mRNA levels of the protooncogenes *MYC*, *FOS*, and *JUN*. However, this effect was consistently more pronounced in the case of HER497R by comparison with HER497K (Fig. 5). Similarly, the effects of TGF-α on the expression of these mRNA moieties was more pronounced in the HER497R-expressing cells (Fig. 5). When two additional HER497R-expressing clones were examined for *MYC*, *FOS*, and *JUN* induction, EGF and TGF-α again exerted a greater effect on the expression of these mRNA moieties by comparison with several HER497K-expressing clones (data not shown).

Parental CHO cells were pleomorphic and flat and did not alter their shape after EGF or TGF-α addition (data not shown). HER497R-expressing cells had a polygonal shape and acquired either a fusiform or a rounded morphology after

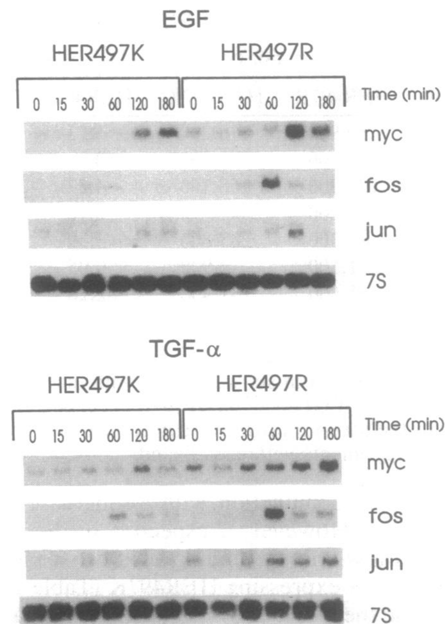

FIG. 5. Effects of EGF and TGF-α on *MYC*, *FOS*, and *JUN* mRNA levels. HER497R- and HER497K-expressing cells were incubated with 1.7 nM EGF or TGF-α for the indicated times. RNA was isolated and analyzed by Northern blotting. Exposure times: *MYC*, 48 hr; *FOS*, 96 hr; *JUN*, 24 hr; 7S, 16 hr. Each autoradiograph is representative of at least three experiments.

addition of EGF for 40 hr (Fig. 4 *C* and *D*). HER497K-expressing cells had a uniformly narrow and fusiform shape in the absence of EGF (Fig. 4*E*), with many cells acquiring a rounded morphology after incubation with EGF for 40 hr (Fig. 4*F*). Similar morphological differences were observed with TGF-α as well as in two other HER497R- and HER497K-expressing clones (data not shown).

DISCUSSION

In the present study, we expressed variant (HER497K) and wild-type (HER497R) EGFRs in CHO cells that are devoid of endogenous EGFRs and compared the two receptors with respect to ligand binding, growth stimulation, tyrosine kinase activation, and induction of *MYC*, *FOS*, and *JUN*. There were two major differences in the binding characteristics of HER497R- and HER497K-expressing CHO cells. First, irrespective of whether binding was performed at pH 7.4 or 8.0, there were no high-affinity binding sites for ^{125}I-TGF-α with HER497K. In contrast, similar high- and low-affinity binding sites were obtained with ^{125}I-EGF and ^{125}I-TGF-α in the case of HER497R and with ^{125}I-EGF in the case of HER497K. Second, at high pH levels, TGF-α dissociation was more pronounced with HER497K by comparison with HER497R. Thus, a single amino acid substitution in subdomain IV of EGFR selectively alters TGF-α binding and dissociation, even though the EGF/TGF-α binding region is located in a 47-amino acid sequence (residues 321–367) within subdomain III (residues 313–446) of EGFR (6, 7). Inasmuch as EGFR dimers appear to have a higher ligand binding affinity than EGFR monomers (17), our findings raise the possibility that the arginine-to-lysine substitution may result in attenuated TGF-α-induced HER497K dimerization. They also imply a modulatory role for this EGFR region on the TGF-α binding pocket within subdomain III.

Tyrosine phosphorylation of specific amino acids in the intracellular domain of EGFR allows for recruitment of important regulatory intracellular substrates such as Shc and Grb2, activation of p21^{ras}, initiation of a cascade of phos-

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phorylation reactions that activate cytosolic proteins, and activation of nuclear oncoproteins (2, 18). In this study, EGF and TGF-α efficiently increased tyrosine phosphorylation of HER497R and HER497K, indicating that the intrinsic tyrosine kinase activity of both receptors was activated by both ligands. However, in HER497R-expressing cells, EGF and TGF-α significantly increased cell growth and caused the anticipated increases in *MYC, FOS*, and *JUN* mRNA levels. In contrast, in HER497K-expressing cells, both factors exhibited a markedly reduced growth response and a decreased ability to raise *MYC, FOS*, and *JUN* mRNA levels. These differences indicate that, in spite of relatively similar intrinsic tyrosine kinase autophosphorylating activities, HER497K activates a less mitogenic pathway than HER497R. Therefore, it is possible that the region in the extracellular domain of EGFR that contains the arginine-to-lysine substitution somehow modulates the efficiency of recruitment of intracellular substrates to the activated receptor and/or allows for the activation of alternative signaling pathways that do not lead to efficient nuclear protooncogene induction and subsequent growth stimulation. The hypothesis that this portion of EGFR may represent an important regulatory region is supported by the observations that the seven residues upstream of codon 497 and the five downstream residues are conserved in the mouse, rat, and human EGFRs (19–21) and that the intercysteine spacing is also completely conserved in this region of the human, rat, mouse, and chicken EGFRs as well as in *let-23*, the homologue of EGFR in *Caenorhabditis elegans* (22).

EGF and TGF-α generally exert the same effects on cell proliferation and function in a variety of cell types. However, a number of quantitative and qualitative differences have been reported between their biological actions (9, 23, 24). In the present study, HER497K-expressing cells were markedly growth inhibited by high concentrations of TGF-α but not EGF, perhaps because of the absence of high-affinity TGF-α binding sites in these cells. Neither factor inhibited the growth of HER497R-expressing cells. Therefore, our findings raise the possibility that some of the differences between the actions of EGF and TGF-α may be due to the presence of HER497K or other variant EGFRs in these cells. In support of this hypothesis, TGF-α exerts a more potent growth inhibitory effect than EGF in RL95-2 human endometrial cancer cells (23), which express both HER497R and HER497K (8).

Recently, several null alleles that result in loss of *let-23* function have been identified (22). Two of these alterations are located in subdomain IV and involve mutations of cysteine to either tryptophan or tyrosine at codons 700 and 753, respectively (22). It is unlikely that these null mutants are analogous to the variant human EGFR described in the present study, inasmuch as HER497K kinase activity was intact and cell proliferation and nuclear protooncogene induction were regulated by this variant receptor in response to ligand binding. In addition, both HER497K and HER497R altered the flattened, nontransformed morphology of the parental CHO cells. Taken together, our data suggest that the extracellular juxtamembranous region of human EGFR has the potential to modulate both ligand binding and transmembrane signaling to the intracellular domain, which may allow the EGF family of ligands to differentially regulate a variety of cellular processes.

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