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Ligand Binding Characterization and Molecular Analysis of Distinct Epidermal Growth Factor-Urogastrone Receptors in Cultured Smooth Muscle and Epithelial Cells from Guinea Pig Intestine

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SUMMARY

In parallel, we measured the receptor binding affinities for epidermal growth factor-urogastrone (EGF-URO) and transforming growth factor-α (TGF-α) in cultured smooth muscle (GCM) and epithelial (GPC) cells derived from guinea pig intestine. The relative order of binding affinities in the GCM cells was TGF-α > EGF-URO, in keeping with the relative order of biological potencies of these polypeptides in a guinea pig gastric circular muscle contractile bioassay. These data established by ligand binding criteria the presence of a TGF-α-preferring receptor in the guinea pig. In contrast, there was a reversed order of binding affinities (EGF-URO > TGF-α) for the polypeptides in GPC cells, in accord with an identical order of bioassay potencies previously observed in a guinea pig gastric longitudinal muscle contractile bioassay. Using a reverse transcription-polymerase chain reaction approach, we also cloned and sequenced putative EGF-URO receptor ligand binding domain III from each cell type. Although the binding specificity for TGF-α and EGF-URO differed in the GCM and GPC cells, the amino acid sequences of receptor domain III were identical in the two cell types. We conclude that the previously measured differences in biological potencies of EGF-URO and TGF-α in the contractile bioassay preparations are due to the distinct receptor binding affinities of EGF-URO and TGF-α that can be detected in different tissues. However, our data document that the distinct relative binding affinites for EGF-URO and TGF-α that can be observed in different cell types from the same species cannot be accounted for solely by the sequence of putative receptor ligand binding domain III.

EGF-URO, first isolated from human urine (1) and from mouse submaxillary glands (2), is an acid-inhibitory/mitogenic polypeptide of approximately 6 kDa that is representative of an enlarging superfamily of structurally related 'growth factors,' including TGF-α, amphiregulin, heparin-binding EGF, and betacellulin (3–8). EGF-URO is known to act by regulating a specific high affinity receptor that possesses intrinsic tyrosine kinase activity and that is closely related to the v-*erb*B oncogene (9–13). Although four members of the c-*erb*B-related group of receptor tyrosine kinases have now been identified (14–17), only the EGF-URO receptor (c-*erb*B) is able to bind EGF-URO and its closely related family members with specificity and high affinity. The restriction enzyme analysis of genomic DNA suggests that there is only a single gene for the EGF-URO receptor (10).

In contrast to the insulin-related family of polypeptides (insulin, insulin-like growth factor I, and insulin-like growth factor II), wherein unique receptors have been found to bind each member of the family with considerable specificity, receptor subtypes that display a preference for individual members of the EGF-URO-related family have yet to be described in molecular terms. Nonetheless, according to conventional bioassay procedures, distinct potencies for EGF-URO and TGF-α have been observed in a variety of contractile smooth muscle systems, including a unique preparation obtained from guinea pig gastric tissue (18). In brief, contractile bioassays showed (18) that the guinea pig gastric LM strip was more sensitive to either human or murine EGF-URO than to human TGF-α, whereas the reverse was true for the CM elements present in

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ABBREVIATIONS: EGF, epidermal growth factor; URO, urogastrone; GCM cells, guinea pig gastric circular muscle-derived smooth muscle cells; GPC cells, guinea pig intestine-derived epithelial cells; CM, circular muscle; DMEM, Dulbecco's modified minimal essential medium; LM, longitudinal muscle; RT, reverse transcription; PCR, polymerase chain reaction; TGF-α, transforming growth factor-α; bp, base pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

the same tissue strip (i.e., the potency of EGF-URO is greater than that of TGF-α in the LM preparation, whereas the reverse is true in the CM tissue). The TGF-α-preferring receptor present in the guinea pig CM tissue reflects an unusual order of potency, which has been observed to date only for the avian c-*erb*B receptor (19). In contrast, the EGF-URO-preferring receptor, present in the LM tissue, reflects the usual order of potencies for human and murine EGF-URO and TGF-α observed in a variety of other mammalian cell systems (20). Our work on the structure-activity relationships for EGF-URO and TGF-α has revealed the importance of the carboxyl-terminal domain of these peptides for receptor binding and for distinguishing between the pharmacological EGF-URO receptor subtypes that can be discerned in the guinea pig gastric smooth muscle tissue (18, 21).

A number of independent approaches, including targeted monoclonal antibodies (22), affinity cross-link-labeling (23), and the generation of chicken/human receptor chimeras (24), have been used to localize the portion of the receptor that binds EGF-URO. Receptor domain III, comprising approximately amino acid residues 310–474 of the human sequence and flanked by two cysteine-rich sequences of the extracellular portion of the receptor, has been found to be responsible for ligand binding. Additionally, domain III has been demonstrated to confer ligand specificity, in that the exchange of this domain in the TGF-α-preferring avian receptor with domain III of the EGF-URO-preferring human receptor yields a chimeric avian/human receptor that binds both EGF-URO and TGF-α with affinities reflecting those of the wild-type human receptor (24).

Based on the observations of Lax *et al.* (24), we hypothesized that the reversed order of biological potencies for EGF-URO and TGF-α in the guinea pig LM and CM preparations might be due to the presence, in selected guinea pig tissues, of an EGF-URO receptor that has distinct binding properties due to alterations in domain III, with one cell type (e.g., CM) containing a receptor reflecting the avian receptor and another cell type having a receptor that more closely reflects the human domain III receptor sequence. Differential splicing of the receptor exons, which has been observed in other receptor systems (25), could readily yield such a situation for the EGF-URO receptor. An alternative hypothesis to account for the differential orders of biological potencies for EGF-URO and TGF-α in the intact CM and LM preparations would be that differences in receptor signaling pathways might in some way change the relative biological potencies for the peptides, as measured in intact cells by bioassay procedures (26). In view of the first of the two hypotheses outlined above, we initally searched for cultured guinea pig-derived intestinal cell strains that would exhibit differences in their ability to bind TGF-α and EGF-URO. We were successful in obtaining a pure culture of smooth muscle cells from the CM tissue (27) but were unsuccessful in obtaining comparable cell cultures from the LM tissue. As an alternative, we sought a pure intestine-derived epithelial cell line for comparison with the CM-derived smooth muscle cells. In this report, we describe two distinct guinea pig-derived cell types that do possess differences in their abilities to bind TGF-α and EGF-URO, firstly, a cell strain (GCM) derived from primary cultures of gastric CM (27) and, secondly, an established cell line (GPC) derived from intestinal epithelium. In parallel, we have used EGF-URO and TGF-α as receptor probes to measure the relative binding affinities of TGF-α and EGF-URO for these cell types, and we have, using a RT-PCR approach, cloned and sequenced the receptor ligand binding domain III from each cell type. Our data show that, although the GCM cells bind TGF-α with a higher affinity than EGF-URO, whereas the converse is true for the GPC cells, both cell types yield the same cDNA sequence for receptor domain III.

Materials and Methods

Cell cultures. Primary smooth muscle cell cultures (GCM), staining positively with the anti-smooth muscle actin antibody HHF35 (28), were obtained from the isolated CM layer of guinea pig gastric tissue as described previously (27). These cultures were homogeneous in terms of their smooth muscle phenotype and did not appear to be contaminated with other cell types (27). It did not prove possible to obtain gastric LM elements, free from CM, to obtain pure gastric LM cell strains. The GCM cells were routinely propagated in 80-cm^2 T flasks, seeded at about 2×10^4 cells/ml in 20 ml of DMEM supplemented with 5% (v/v) fetal calf serum. GCM cell strains obtained from individual animals were used for up to 10 generations and were subcultured at 1-week intervals at a passage ratio of 1:4 (each passage leads to two population doublings). In our search for a guinea pig cell strain distinct from the smooth muscle GPC cells, we decided to evaluate intestine-derived epithelial cells. The guinea pig colonic epithelial cell line selected (GPC) was obtained from the American Type Culture Collection (catalogue number CCU242) and was propagated as a homogeneous-appearing cell line in DMEM/5% fetal calf serum as for the GCM cells, with a passage ratio of 1:10. For binding studies, as described in further detail below, cells were subcultured and grown to confluency in 3.5-cm Nunclon multidish trays (GIBCO, Grand Island NY).

Peptides and other reagents. Murine EGF-URO was isolated from the submaxillary glands of testosterone-treated male mice (29). The polypeptide yielded a single peak when analyzed by reverse phase liquid chromatography (C_{18} column) and yielded a single band upon gel electrophoresis. In a mitogenesis assay using human fibroblasts, the EC_{50} for this peptide was routinely ~0.25 ng/ml (40 pM) (20, 30). Human recombinant TGF-α, expressed in *Escherichia coli* and purified by gel filtration and high performance liquid chromatography as described (5, 31), was kindly provided by Dr. M. Winkler (Genentech, San Francisco, CA). On a weight basis, using a mink lung cell (CCL 64) radioreceptor assay, TGF-α prepared in this manner exhibits a specific activity of 0.55 ng of EGF-URO receptor equivalents/ng of TGF-α (31). The concentrations of stock solutions of EGF-URO dissolved in 50 mM sodium bicarbonate and diluted in phosphate buffer, pH 7.4, were measured spectrophotometrically, as done previously (20), using the formula $(E_{215} - E_{225}) \times 155 = \mu$g/ml. These values agreed exactly with measurements of peptide concentration obtained by amino acid analysis of stock solutions. Because of the limited amounts of peptide available, the concentration of TGF-α in the stock solution was determined only by amino acid analysis. Carrier-free ^{125}I and ^{35}S-labeled deoxynucleotides were from Amersham Canada (Oakville, Canada).

Isolation and sequencing of receptor cDNA clones. Either the CM (GCM) or colonic epithelial (GPC) cells were grown to confluence in 80-cm^2 T flasks, and total cellular RNA was isolated using either a modified guanidinium thiocyanate/cesium trifluoroacetic acid method (32) or the TRI reagent (Molecular Research Center, Cincinnati, OH). The RNA so isolated (1 μg) was reverse-transcribed with superscript reverse transcriptase (BRL) and oligo(dT)$_{12-18}$ primer, according to the method of Rappolee *et al.* (33, 34), at 42° for 60 min, followed by denaturation at 93° for 5 min and flash-cooling to 4°. The RT product was then used with sequence-specific primers (0.1 μM) (see Table 3) for PCR amplification using 1 unit of AmpliTaq polymerase in 10 mM Tris·HCl buffer, pH 8.3 (0.1-ml final volume), containing $MgCl_2$ (2.5 mM), KCl (50 mM), nuclease-free bovine albumin (5 μg), and each of the four deoxynucleotide triphosphates (0.6 μM levels each). Amplification was allowed to proceed for 60 cycles, beginning with a 1-min

denaturation period at 94°, followed by a 2-min reannealing time at 55° and a primer extension period of 2 min at 72°. The PCR products were purified by 2% agarose gel electrophoresis and cloned in the pBluescript KS(+) or SK(+) vector (Stratagene), followed by transformation of *E. coli* strain JM 109 cells (Stratagene). The insert was sequenced in both strands with M13 universal and reverse sequencing primers, using the dideoxynucleotide sequencing method (35), employing either a T7 DNA polymerase sequencing kit (Pharmacia) or the Sequenase 2.0 kit from United States Biochemical Corp. (Cleveland, OH).

The three sets of PCR primer pairs (see Table 3), two of which were designed to yield *Eco*RI, *Xho*I, and *Hind*III restriction sites, were based on consensus oligonucleotide sequences found in the human, rat, and avian EGF-URO receptor domain III cDNAs (10, 19, 36) and were synthesized with the assistance of Dr. R. C. Pon of the Regional DNA Synthesis Laboratory, University of Calgary, Faculty of Medicine.

Measurements of ligand binding. ^{125}I-labeled EGF-URO was prepared by the chloramine-T/metabisulfite method essentially as described previously (20, 37), except that radiolabeled EGF-URO (190–350 cpm/pg) was separated from the reaction mixture by chromatography in 0.1 M phosphate buffer, pH 7.4, containing 0.1% (w/v) bovine serum albumin, on a column of Sephadex G-10 (10-ml bed volume, in a 10-ml disposable pipette) (38). This method of radiolabeling has been shown to yield ^{125}I-EGF-URO that is fully biologically active in a fibroblast thymidine incorporation assay and that in our hands yields highly reliable ligand binding data (20, 38). Using the same radiolabeling protocol, we were not able to obtain ^{125}I-TGF-α that yielded reproducible specific binding to the GPC and GCM monolayers, even though the radiolabeled reaction product exhibited good specific binding to human placenta membranes. The binding of ^{125}I-EGF-URO to replicate, intact, rinsed, confluent GCM and GPC cell monolayers, grown in 3.5-cm-diameter multidish trays, was measured at 4° in DMEM supplemented with 1 mg/ml bovine albumin, 0.1 μM KI, and 20 mM HEPES buffer, pH 7.4. Experiments were done with GCM and GPC cell monolayers grown in parallel. Binding equilibrium was established for 5 hr, at which time monolayers were rapidly rinsed free of unbound radioligand with ice-cold phosphate-buffered saline, pH 7.4, containing 1 mg/ml bovine albumin and 0.1 μM KI. During this time course at 4°, the radioligand was essentially undegraded, as evidenced by the high degree of precipitability with 10% (w/v) trichoroacetic acid. Rinsed monolayers were solubilized in 1 ml of 1 N NaOH and radioactivity in the entire sample was measured by crystal scintillation counting (approximately 85% efficiency). Parallel cell monolayers were disaggregated with trypsin/EDTA and hemacytometer cell counts were done to determine the average number of cells per monolayer (about 120,000 cells/dish). For the binding of ^{125}I-EGF-URO at increasing radioligand concentrations, specific binding was defined as the difference between the total amount of radioactivity bound and the amount bound in the presence of an excess (≥500-fold) of unlabeled EGF-URO. Nonspecific binding usually accounted for <15% of the total amount of radioactivity bound. Binding data were analyzed either graphically or by a curve-fitting program (39) (EBDA/LIGAND) designed for the analysis of ligand binding data, essentially according to the method of Scatchard (40). Values obtained graphically from the binding curves (maximum binding and half-maximal saturation of binding) were entirely in accord with estimates calculated by the EBDA/LIGAND program. For binding competition experiments with unlabeled EGF-URO and TGF-α, GCM and GPC monolayers were grown in parallel to comparable cell densities with the same growth medium and serum batch. Binding competition experiments for the two sets of monolayers were routinely done in parallel on the same day, with the same set of labeled and unlabeled peptide reagent stock solutions. To validate each set of observations, experiments were repeated over a time period of 2–3 months using separately grown crops of monolayers, so as to yield three or four independently conducted experiments for the estimates of relative binding affinities. GCM and GPC monolayers were incubated at 4° for 5 hr with the identical concentration of radiolabeled EGF-URO (3 ng/ml, 0.5 nM) in the presence or absence of increasing concentrations of either unlabeled EGF-URO or TGF-α. At equilibrium (5 hr), cells were rinsed and solubilized as described above for the measurement of specifically bound ^{125}I-EGF-URO.

Results

Binding of ^{125}I-EGF-URO to GCM and GPC monolayers. The binding of ^{125}I-EGF-URO to both GCM and GPC monolayers at 4° reached a plateau between 4 and 5 hr and remained stable for up to 7 hr (data not shown). Thus, a 5-hr equilibrium time point was selected for the measurement of binding at increasing concentrations of ^{125}I-EGF-URO. As illustrated in Fig. 1, the binding of ^{125}I-EGF-URO was saturable, approaching a maximum in the range of 60–120 ng/ml (10–20 nM) peptide. Analysis of the binding data according to the method of Scatchard (40) yielded a curvilinear plot for both GCM and GPC monolayers (Fig. 1, *insets*), which was interpreted to reflect two classes of EGF-URO binding sites, of higher and lower affinities, in both cell types. Curve-fitting analysis of the data revealed that, for each of the two classes of binding sites, the smooth muscle GCM cell monolayers bound ^{125}I-EGF-URO with about half the affinity exhibited by the epithelial GPC cells (Table 1). Although the total numbers of binding sites for the two cell types were of the same order of magnitude (2.4–7.8 × 10^4 sites/cell), the GPC cells possessed 3–4-fold more receptors/cell, compared with the GCM cells.

Binding competition studies. Both unlabeled EGF-URO and TGF-α competed effectively with the binding of ^{125}I-EGF-URO to both GCM and GPC cell monolayers (Fig. 2). Unrelated disulfide-containing polypeptides (2 μM insulin or 1 μM oxytocin) did not compete with ^{125}I-EGF-URO binding in either cell strain (data not shown). The competition with ^{125}I-EGF-URO binding by unlabeled TGF-α in the GCM cell monolayers yielded a 2-fold lower IC_{50} than did that by unlabeled EGF-URO (Fig. 2; Table 2). That is, the affinity of unlabeled TGF-α for the receptor site in the GCM cells was higher than that of unlabeled EGF-URO. Conversely, in the GPC cells the competition with ^{125}I-EGF-URO binding by TGF-α yielded a higher IC_{50} than did that by EGF-URO, indicating a reversed order of receptor affinities for the peptides in the GPC cells, compared with the GCM cells, as summarized in Table 2. Thus, not only did the two cell types exhibit distinct affinities for ^{125}I-EGF-URO but the GCM and GPC cells also exhibited distinct and reversed relative affinities for unlabeled TGF-α and EGF-URO. Despite the heterogeneity of binding sites revealed by the Scatchard plots, it was still possible to use the binding competition data to calculate, as we have done for EGF-URO previously (20), dissociation constants (K_i values) for unlabeled EGF-URO and TGF-α, according to the equation (41) $K_i = IC_{50}[1/(1 + L^*/K^*)]$, where L^* represents the concentration of radiolabeled EGF-URO present in the competition assay (0.5 nM) and K^* represents the affinity of radiolabeled EGF-URO. For the calculation, the value of K^* was taken as the weighted average (K_w^*) according to the proportion of high and low affinity sites in the GCM and GPC cells (K_w^* = 3.9 nM for GCM cells and 1.4 nM for GPC cells). Using the aforementioned formula and the IC_{50} values, the K_i values calculated for unlabeled EGF-URO in the two cell types were as follows: GCM, 1.1 nM; GPC, 0.4 nM. These values were very close to the K_d values estimated for the high affinity ^{125}I-EGF-URO binding sites by Scatchard plot analysis (Table 1). It can

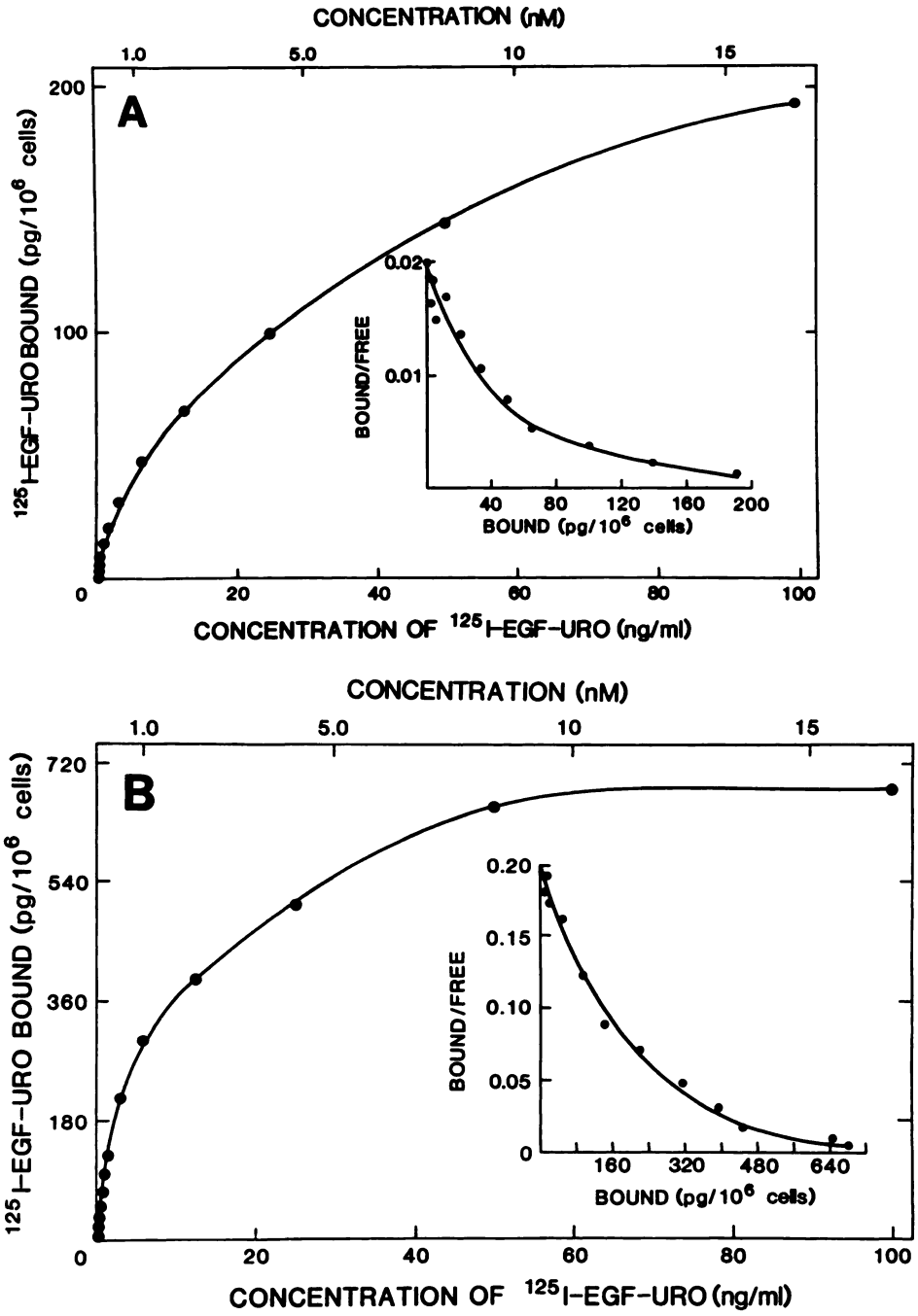


Fig. 1. Binding of ^{125}I-EGF-URO to GCM (A) and GPC (B) cells as a function of ^{125}I-EGF-URO concentration. Multiple, independently grown, confluent cell monolayers were rinsed and incubated for 5 hr at 4° with 1 ml of binding medium containing increasing concentrations of ^{125}I-EGF-URO (specific activity, 210 cpm/pg), either in the absence or in the presence of a 500-fold excess of unlabeled EGF-URO. The amount of cell-associated radioactivity was then determined as described in Materials and Methods. Only the specific binding is shown. Nonspecific binding of radiolabeled EGF-URO to either GCM or GPC cells was <15% of total binding for concentrations of ^{125}I-EGF-URO below 50 ng/ml and approached 40% of total binding at a concentration of 100 ng/ml ^{125}I-EGF-URO. Each data point, corrected for nonspecific binding, represents the average of measurements on three separately grown monolayers. *Insets*, plot of the data according to the method of Scatchard (40). The figure is representative of three independently conducted experiments.

TABLE 1

Summary of Scatchard plot analysis of binding data obtained with ^{125}I-EGF-URO

The binding of increasing concentrations of ^{125}I-EGF-URO to GCM and GPC cell monolayers was measured as illustrated in Fig. 1, and the resulting data were analyzed according to the method of Scatchard (40), as outlined in the text. Values represent the means ± standard errors for binding parameters resulting from three independently conducted binding experiments for each cell type, done with separate replicate crops of cultured monolayers.

Cells	Binding affinity (K_d)		Binding capacity (B_{max})		Receptor number
	High affinity	Low affinity	High affinity	Low affinity	
	nM		*pg/10^6 cells*		*total sites/cell*
GCM	0.8 ± 0.1	4.9 ± 0.7	60	180	2.4×10^4
GPC	0.4 ± 0.1	2.2 ± 0.3	327	450	7.8×10^4

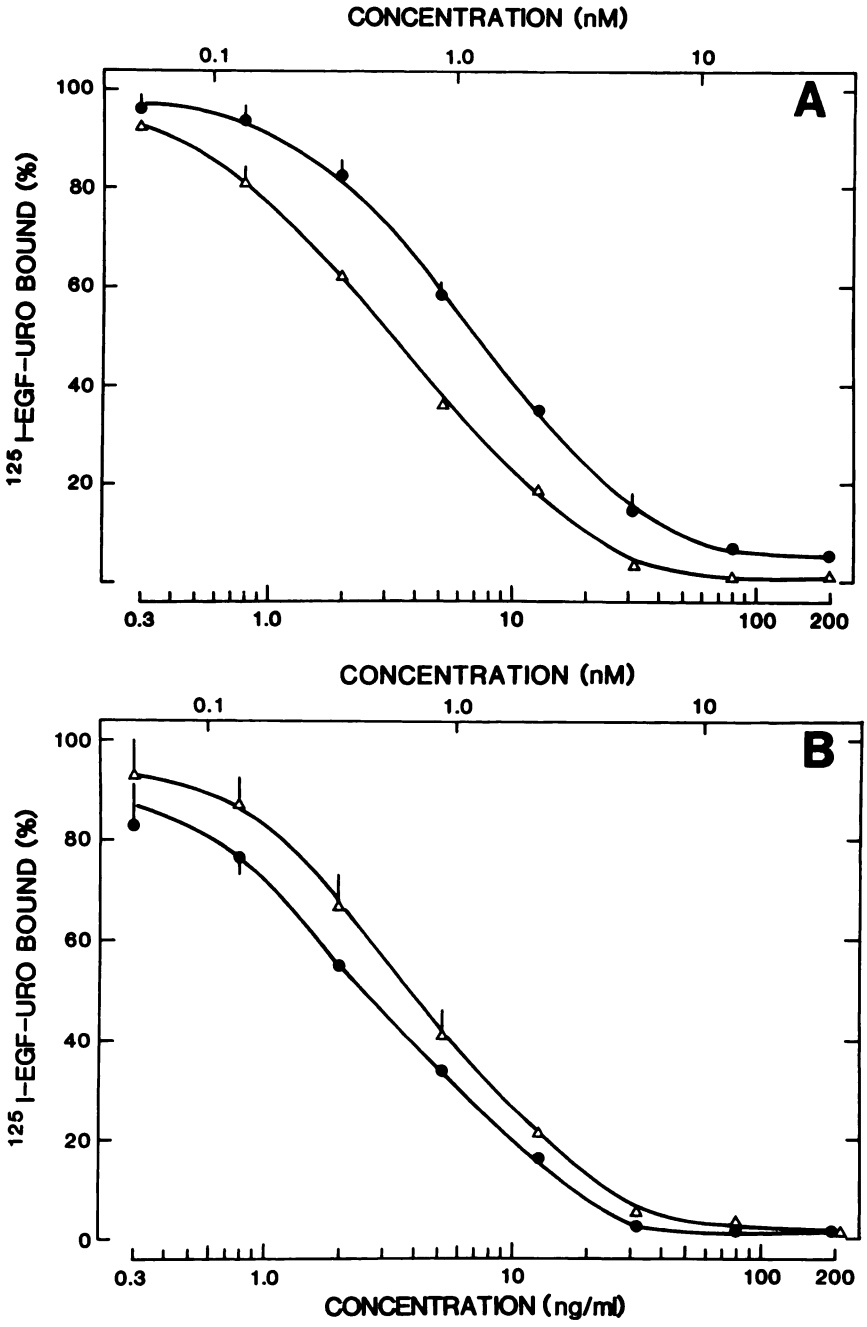


Fig. 2. Competition inhibition of ^{125}I-EGF-URO binding to GCM (A) and GPC (B) cell monolayers by unlabeled EGF-URO and TGF-α. Multiple, independently grown, confluent cell monolayers were incubated for 5 hr at 4° with 3 ng/ml ^{125}I-EGF-URO (350 cpm/pg), in the absence or in the presence of increasing concentrations of unlabeled EGF-URO (●) or TGF-α (△). The net amount of specifically bound radioactivity was then determined as outlined in Materials and Methods. The specific binding in the absence of competitor (2489 ± 110 cpm in GPC cells and 1600 ± 60 cpm in GCM cells) was expressed as 100% and the binding of ^{125}I-EGF-URO at each concentration of unlabeled competitor was calculated relative to the 100% value. Each *point* represents the average ± standard error of measurements made on three independently grown replicate monolayers. The figure is representative of six independent experiments for GCM cells with separately grown crops of monolayers, using cell strains derived from two separate animals (passages 3 and 10), and is representative of four independent experiments for GPC cells with separately grown crops of monolayers.

also be pointed out that the K_i value of 0.4 nM obtained for the GPC cells is remarkably close to the K_i value of 0.3 nM that was estimated previously by the same approach for the binding of unlabeled murine EGF-URO to human fibroblast monolayers (20). Thus, the K_i values calculated for unlabeled TGF-α (0.4 nM for GCM cells and 0.6 nM for GPC cells) probably also reflect accurately the high affinity binding constant for the interaction of unlabeled TGF-α with the receptor. What was clear from both the binding competition curves shown in Fig. 2 and the calculations of peptide affinities was that in terms of ligand binding the GCM cells possess a TGF-α-preferring receptor, whereas the GPC cells possess an EGF-URO-preferring receptor. Unfortunately, it did not prove possible to obtain reliable measurements of the specific binding of ^{125}I-labeled TGF-α in the GCM monolayers because of unacceptable nonspecific binding. Furthermore, the low receptor abundance in the GCM cells did not allow for receptor analysis using a cross-link-labeling approach (38) for a comparative analysis of the GCM receptor with the GPC receptor, which did yield a 180-kDa constituent when cross-link-labeled with ^{125}I-EGF-URO (data not shown). Thus, it was not possible to compare the K_i values for unlabeled TGF-α with those obtained by direct measurements of ^{125}I-TGF-α binding and it was not possible to directly compare the electrophoretic mobility of the cross-link-labeled receptor in the GPC cells with that of the receptor in the GCM cells.

Isolation and sequencing of the cDNA for receptor domain III from GCM and GPC cells. Because the ligand binding experiments demonstrated a differential affinity for TGF-α and EGF-URO between the GCM and GPC cells, we

TABLE 2

Estimates of ligand affinity by binding competition

The IC_{50} values for unlabeled EGF-URO and TGF-α were determined from curves such as those shown in Fig. 2. Values represent the means ± standard errors for six independently conducted binding competition experiments with GCM monolayers and four independently conducted experiments with GPC cell monolayers. The apparent K_i values were calculated from the IC_{50} values according to the method described in the text.

Peptide	IC_{50}		K_i	
	GCM cells	GPC cells	GCM cells	GPC cells
	ng/ml (nM)		nM	
EGF-URO	7.2 ± 0.7 (1.2)[a,b]	2.8 ± 0.3 (0.5)[a,b]	1.1	0.4
TGF-α	3.1 ± 0.4 (0.5)[a]	4.8 ± 0.8 (0.8)[a]	0.4	0.6

[a] $p < 0.01$, for differences between the IC_{50} values for EGF-URO and TGF-α obtained either in GCM or in GPC cells.
[b] $p < 0.01$, for differences in EGF-URO binding between GCM and GPC cells.

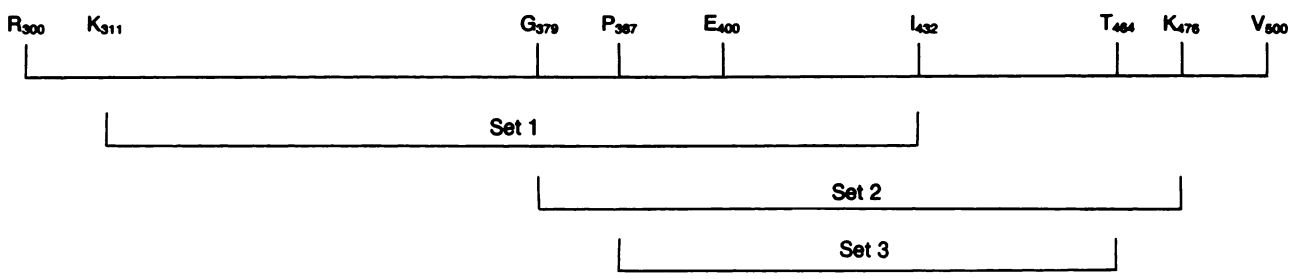
sought to determine the sequence of receptor domain III in each cell type. As summarized above, it is believed that this domain accounts for ligand binding and for the differential specificity of EGF-URO and TGF-α binding observed between the human and avian receptors (19, 24). We first attempted to detect mRNA for the EGF-URO receptor in GCM cells using reduced stringency Northern blot analysis and a radiolabeled cDNA receptor probe comprising the external domain of the human EGF-URO receptor. Because we failed to detect any appreciable signal (data not shown), we concluded that the abundance of receptor message was probably quite low in the GCM cells and that an RT-PCR approach would be better suited to our goal of obtaining the cDNA sequence for receptor domain III from the GCM and GPC cells. Therefore, three sets of overlapping PCR primers were designed (Table 3) to span the domain III region, corresponding to amino acid residues 310–474 of the human receptor sequence. As shown in Fig. 3B, by using primer set 2 robust amplification of a PCR fragment of the expected size (297 bp) was obtained with RNA from human A431 cells, which are known to contain an abundance of human receptor message (10). A comparable 297-bp amplification product was also routinely detected from the epithelial GPC cell-derived RNA, but only a faint band in the 297-bp region was detected from the smooth muscle GCM cell RNA (Fig. 3B). Both GCM and GPC cells also reproducibly yielded a 240-bp PCR product of comparable intensity, but no such product was obtained from A431 cell RNA. Using an intron-spanning β-actin primer pair (42), the RT-PCR procedure yielded a comparably strong, expected, intron-free, 243-bp signal from all three cell types, indicative of the absence of genomic DNA in the RNA preparations (Fig. 3A). Compared with the β-actin signal, the PCR signals obtained with primer set 2 underscored the relatively low abundance in both GCM and GPC cells of mRNA coding for the sequence of receptor

TABLE 3

Oligonucleotide primer pairs

Three sets of overlapping oligonucleotide primer pairs that span the sequence of receptor domain III were synthesized corresponding to the human receptor sequences shown. Degenerate primer sequences were used for set 1; the guinea pig receptor sequence found using primer set 2 was used to design the nested primer set 3. Restriction enzyme sites are shown for sets 1 and 2.

Primer pair	Oligonucleotide sequence												Corresponding human receptor domain III amino acid sequence
Set 1													
5′ primer	AAA	*Hin*dIII A↓AG	CTT	AA(A/G)	GTN	TG(C/T)	AA(C/T)	GGN	ATC/T	GG			K_{311}-G_{317}
3′ primer	GG	GGA↓ *Eco*RI	ATT	CGA	GAT/T	(C/T)TC	(C/T)TT	NAG	NGA	NCG			R_{427}-I_{432}
Set 2													
5′ primer	GGG	TTT	TTG	CTG	ATT	*Hin*dIII CA↓A	GCT	TGG	C				G_{379}-W_{386}
3′ primer	GG	*Eco*RI G↓AA	TTC	CTT	GCA	GCT	GTT	TTC	ACC	TCT	GTT	GCT	TAT	I_{467}-K_{476}
Set 3													
5′ primer	CCT	GAG	AAC	AGG	ACC	AAC	CTC	C						P_{387}-L_{393}
3′ primer	GGT	TTT	CTG	TCC	GGC	AGT	TCC	A						F_{457}-T_{464}



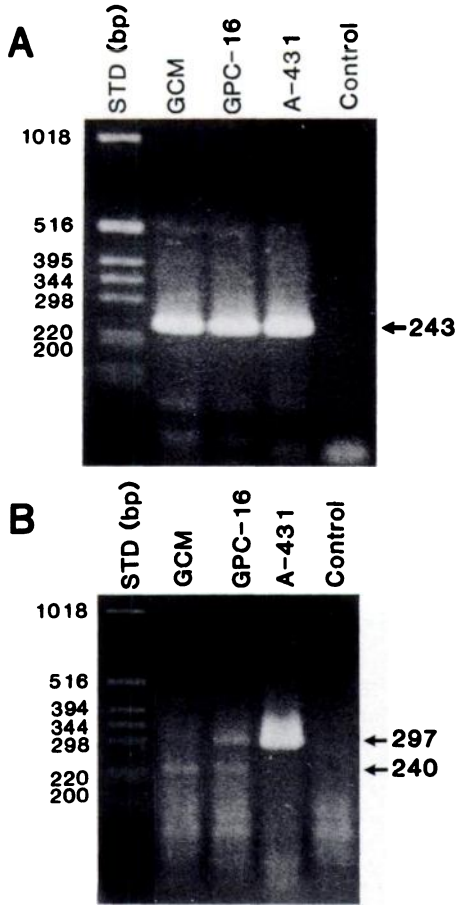


Fig. 3. Detection of mRNA transcripts in GCM, GPC, and A431 cells by RT-PCR using β-actin primers (A) and EGF-URO receptor primer set 2 (B). Total RNA was extracted from confluent GCM, GPC (strain 16), and A431 cells and was reverse transcribed into cDNA as described in Materials and Methods. The samples were amplified by 60 cycles (each cycle consisted of 1 min at 94°, 2 min at 55°, and 2 min at 72°) of PCR either with primer set 2 (B) (see Table 3) or with a previously described actin primer pair (42). Control samples contained no added RT product. Molecular weight marker positions (1-kilobase ladder) (*STD*) are shown on the *left*; the values on the *right* (*arrows*) show the positions expected for the estimated sizes of the PCR products anticipated from the actin primer pair and from EGF-URO receptor primer set 2. An unexpected 240-bp PCR product from the GCM and GPC cells is also indicated.

domain III. The data also suggested that another receptor-related cDNA, yielding the 240-bp sequence, might be present. However, preliminary analysis of the 240-bp product yielded a sequence unrelated to the EGF-URO receptor but showing 70–80% homology with the 28 S rRNA gene from several species (5′-CCCCCGGGCTGCTGGCATCACTTATCTCCTCGAG-GGAGCGGGAAGGGGTTCGGAGTCGGTGGTTGGGGGG-GGGGTCCTCCTCCCCGGGGCCGGCCCCGGACGCGTGT-CGCCCCGCGACGCTACGCCGCGACGAGTAGGAGGGGC-CGCTGCGTGAGCTTGAAGCTAGGGGCCGGGCGGTGA-GCGCGCAGTGCAGATCTTTT-3′). This sequence was not investigated further. The 297-bp fragment did, nonetheless, yield a sequence highly homologous to the human EGF-URO receptor (see Fig. 4). Routinely, the PCR signals for the 297-bp receptor sequence yielded by the GCM cell-derived RT product were quite low, presenting considerable difficulty for the successful recovery of cloned inserts in the pBluescript vector despite repeated attempts. For that reason, we decided not to proceed beyond the sequencing of receptor domain III, which was our primary goal. With the use of the overlapping primer sets 1 and 2, along with confirmation of the sequences by the use of the nested primer set 3, the identical, composite, EGF-URO receptor nucleotide sequence of 448 bp was obtained from both GCM and GPC cells, as shown with the deduced amino acid sequence in Fig. 4. The composite sequence was obtained from four independently sequenced clones obtained from two independently prepared RT-PCR products from GCM cells and from 15 independently sequenced clones obtained from three independently prepared RT-PCR products from GPC cell RNA.

Discussion

Based on our previous structure-activity work (18), which revealed differential biological activities of EGF-URO and human TGF-α in guinea pig smooth muscle contractile bioassay systems, we selected murine EGF-URO and human TGF-α as the two best receptor probes for our ligand binding study, because these peptides were able to discriminate reliably between the receptor systems in the guinea pig LM and CM preparations and in other assay systems we have used (20, 43). We observed that human and murine EGF-URO are essentially equipotent in the smooth muscle bioassay (18). Unfortunately, guinea pig EGF-URO and TGF-α have not been available for our work. One of the main findings of our study was that the same two ligands that had been previously characterized by us for their distinct relative biological potencies in the guinea pig bioassay systems also showed distinct and reversed relative affinities in the ligand binding assays done in the smooth muscle GCM cells and the epithelial GPC cells. We believe it is significant that the peptide binding specificity of the TGF-α-preferring receptor in the smooth muscle-derived GCM cells is exactly in accord with the order of biological potencies (TGF-α > EGF-URO) measured for TGF-α and EGF-URO in the contractile bioassay using the guinea pig CM strip preparation (18). A comparable order of peptide potencies has also been observed for TGF-α and EGF-URO in a bovine coronary artery assay (43). Importantly, this order of binding affinities is also in complete agreement with the relative biological potencies (TGF-α > EGF-URO) that we observed previously for the two polypeptides in stimulating [^{3}H]thymidine incorporation in GCM cells (27). Thus, one conclusion that can be drawn from our work is that the relative biological potencies that we observed previously for EGF-URO and TGF-α analogues by bioassay procedures do very likely reflect the relative binding affinities of the polypeptides at the receptor site. To our knowledge, our work represents the only documentation to date, using both bioassay and binding criteria, of the presence of a TGF-α-preferring receptor in a mammalian species that also exhibits an EGF-URO-preferring receptor (as described in the following paragraph).

In contrast to the GCM cells, the peptide binding specificity of the EGF-URO-preferring receptor in the colonic GPC cells reflects accurately the comparable order of peptide potencies observed in the guinea pig gastric LM contractile bioassay (18). This order of peptide potencies (EGF-URO > TGF-α), which is taken as the norm for mammalian EGF-URO receptors, has also been observed by us in a porcine coronary artery contractile assay (43) and was observed by us with comparable reagents many years ago in a human fibroblast thymidine incorporation assay (20). Unfortunately, because of the high level of back-

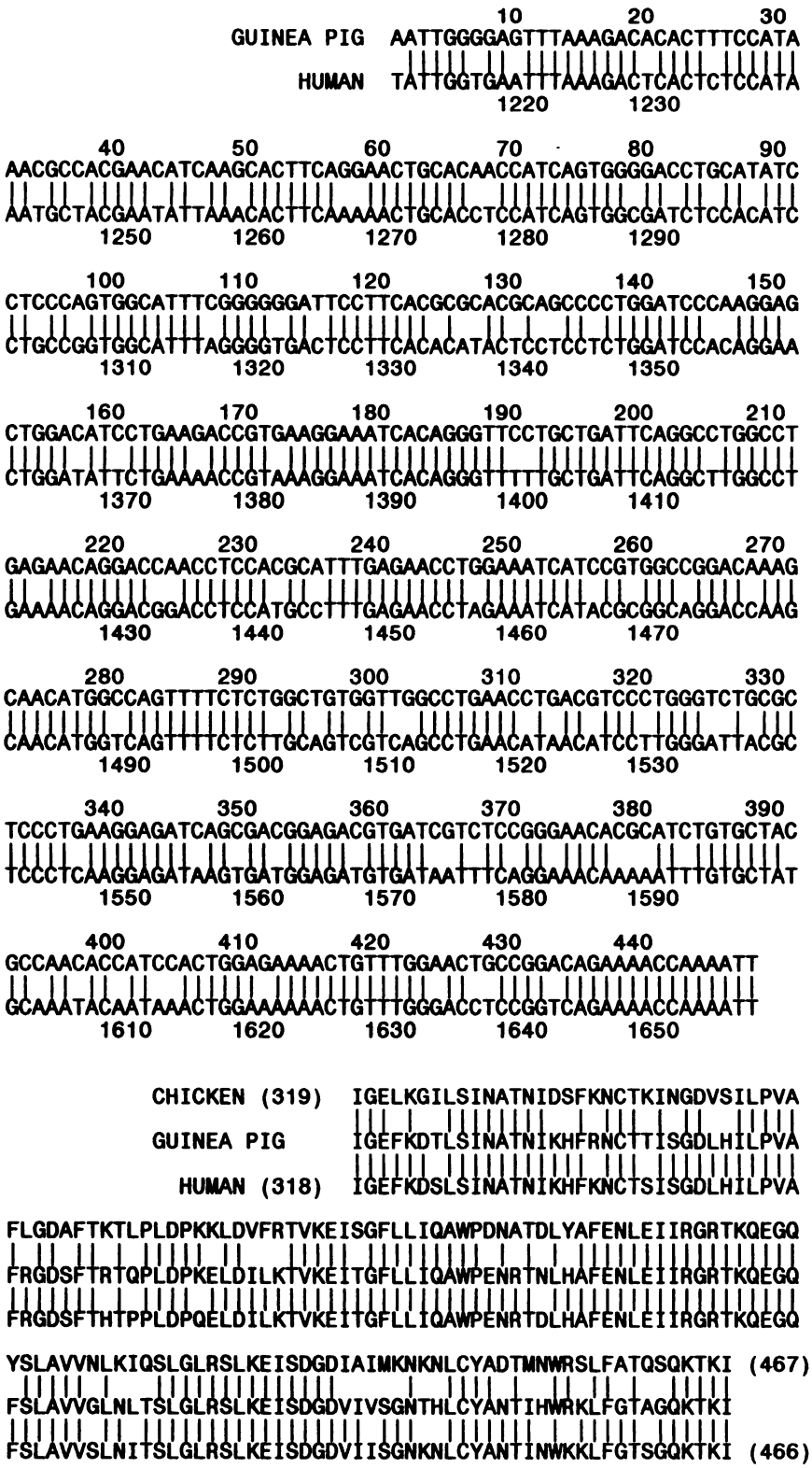


Fig. 4. Oligonucleotide sequence (*upper*) and deduced amino acid sequence (*lower*) of guinea pig GCM/GPC cell EGF-URO receptor domain III. The oligonucleotide sequence of the guinea pig receptor (*upper*) is shown with the oligonucleotide sequence of the human receptor, numbered as published previously (10). The deduced amino acid sequence of guinea pig receptor domain III (*lower*) is compared with the sequences in the human and chicken receptors. The numbering of the amino acid residues (chicken, I_{319} to L_{467}; human, I_{318} to L_{466}) is in accord with the previously published sequences (10, 19). *Vertical bars*, sites of identity between the guinea pig receptor and the other two receptors.

ground [^{3}H]thymidine incorporation in the colonic GPC cells, it did not prove possible to determine relative biological potencies for EGF-URO and TGF-α in a mitogenesis assay with this cultured cell system. Nonetheless, the GPC cells, constituting a continuous cell line, as opposed to the tissue-derived GCM cell strain, can be taken as representative, in terms of relative binding affinities, of a wide spectrum of previously studied cell and tissue types, including the guinea pig LM preparation.

Differences in EGF-URO/TGF-α peptide potencies between species (e.g., porcine versus bovine artery or human versus avian fibroblasts) might readily be attributed to species differences in the receptors *per se*, as has been documented for the avian receptor (19). Such species differences could not, however, account for the potency differences observed in the LM and CM preparations coming from the same tissue of the same species (i.e., guinea pig gastric LM versus CM). Classically,

such potency differences are taken to reflect the presence of receptor subtypes (44). The distinct peptide binding specificities that we have observed for the guinea pig GCM (TGF-α > EGF-URO) and GPC (EGF-URO > TGF-α) cells in the present study represent the ligand binding equivalent of the receptor subtypes that we previously detected by bioassay in the LM and CM preparations from this species. Our data thus support the hypothesis that the distinct order of biological potencies previously observed in the gastric LM and CM bioassays (18) is due to a distinct order of receptor ligand binding specificity, rather than a pharmacodynamic receptor-coupling mechanism in some way related to the signal transduction process (26). Unfortunately, we were unable to isolate pure smooth muscle populations from the gastric LM elements to determine whether these 'LM'-derived cells might parallel the binding specificity of the colon-derived epithelial cells, so as to be distinct from the TGF-α-preferring CM-derived smooth muscle cells.

Given the reversed TGF-α/EGF-URO binding specificity of the GCM and GPC cells, we were surprised to discover that the cDNA sequences of putative ligand binding domain III were identical in the two cell types. Overall, the translated guinea pig domain III amino acid sequence is much more homologous to the human (about 90% homology) than to the avian (about 72% overall homology) receptor sequence. The finding that the guinea pig receptor does bind murine EGF-URO with high affinity (K_d ~ 0.4–5 nM), compared with the binding of EGF-URO to the avian receptor (K_d ~ 260 nM), is in keeping with the closer homology of the guinea pig receptor domain III to the human domain III receptor sequence. Remarkably, the affinity of the GPC receptor for unlabeled EGF-URO, measured in the present study by the binding competition assay (K_i = 0.4 nM), was virtually the same as the affinity of unlabeled EGF-URO for primary cultures of human fibroblasts (K_i = 0.3 nM) that we measured by the same methods some time ago (20). Furthermore, it can be noted that the affinities of the GPC and GCM receptors that we have observed for TGF-α (K_i values of 0.4–0.7 nM) are close to the affinities for TGF-α reported by Lax *et al.* (19, 24) for the human (K_d = 1.8 nM) and avian (K_d = 0.9 nM) receptors. Thus, the differences in the domain III receptor sequences, between the more closely related guinea pig and human receptors on the one hand and the avian receptor on the other, do not appear to involve those receptor residues responsible for binding TGF-α. Rather, the domain III sequence differences between the avian receptor and the other two mammalian receptors appear to involve receptor amino acids that may interact specifically with complementary amino acid residues present in EGF-URO and not in TGF-α. Previously (18, 20), we pointed to the importance of the carboxyl-terminal sequence in EGF-URO (WWELR) for ligand affinity. This sequence has no counterpart in the sequence of TGF-α or in the other members of the EGF-URO family. Very possibly the WWELR motif in the EGF-URO sequence may be found to interact in a unique way with a complementary region of domain III in the human and guinea pig receptors, so as to confer high affinity peptide binding, compared with the avian receptor.

Our results are in keeping with the conclusion that the sequence of receptor domain III is important for high affinity EGF-URO binding. Nonetheless, our sequence data do not explain the distinct relative binding specificities of the GCM and GPC cells for EGF-URO and TGF-α. One clear indication that can be drawn from our work is that, in contrast to the implications of work with the domain III sequence of the avian EGF-URO receptor (19), indicating that this domain can confer TGF-α binding specificity, receptor domain III alone does not appear to be sufficient for determining the differential affinities of the receptor for EGF-URO and TGF-α. Possibly there may be minor sequence differences outside the ligand binding domain in the EGF-URO receptors of the GCM and GPC cells that may account for the differential ligand specificity of the two cell types. Alternatively, it is possible that post-translational modification of the same guinea pig receptor sequence (for example, differential glycosylation of the extracellular domain), on the one hand in GCM cells and on the other hand in GPC cells, may account for the receptor binding subtypes that we have described. Precedent for such a situation can be found in results with the cloned bradykinin receptor, which exhibits two distinct receptor binding subtypes upon cellular expression in COS cells (45). Unfortunately, because of the low abundance of receptor message in the GCM cells, we have not yet been able to isolate full-length receptor clones for a detailed comparative evaluation of the receptor sequences present in GCM and GPC cells. Continuing work in our laboratory is aimed at the goal of obtaining such full-length guinea pig receptor clones for further study.

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