MOLECULAR PHARMACOLOGY, 17, 314–320

Epidermal Growth Factor–Urogastrone: Biological Activity and Receptor Binding of Derivatives[1]

MORLEY D. HOLLENBERG AND HAROLD GREGORY*

*Howard Hughes Medical Institute, Division of Clinical Pharmacology, Departments of Medicine and of Pharmacology & Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and *Pharmaceuticals Division ICI Ltd., Mereside, Alderley Park, Macclesfield, Cheshire, England SK10 4TG*

Received May 29, 1979; Accepted January 10, 1980

SUMMARY

HOLLENBERG, M. D., AND H. GREGORY. Epidermal growth factor–urogastrone: Biological activity and receptor binding of derivatives. *Mol. Pharmacol.* 17: 314–320 (1980).

We have examined the receptor binding (cultured human fibroblasts) and biological activities (stimulation of human fibroblast thymidine incorporation; inhibition of canine gastric acid secretion) of both human and mouse epidermal growth factor–urogastrone (EGF-URO) and of derivatives of these 53 amino acid polypeptides lacking up to six of the C-terminal amino acid residues. Both the human and the mouse polypeptides are biologically equipotent, although human EGF-URO possesses a two- to threefold higher human fibroblast receptor affinity than does mouse EGF-URO; binding kinetic methods designed to assess the receptor affinity of the unlabeled peptides indicate that the ^{125}I-labeled peptides exhibit a lower fibroblast receptor affinity compared with native peptides. Whereas the shortened peptides (EGF-URO 1–47) are equipotent with the intact molecule (EGF-URO 1–53) in inhibiting gastric acid secretion, both mouse and human EGF-URO 1–47 are only one-tenth as potent as EGF-URO 1–53 in stimulating fibroblast thymidine incorporation; human EGF-URO 1–52 is equipotent with the intact peptide. Neither the human C-terminal hexapeptide (EGF-URO 48–53) nor the 48–52 pentapeptide interacts with the receptor. The reduced carboxamidated intact polypeptide is likewise inactive. In the presence of an excess of the C-terminal EGF-URO 48–53, EGF-URO 1–47 does not exhibit an increased biological potency. The reduced biological potencies of the shortened derivatives (EGF-URO 1–47) in fibroblasts are paralleled by reduced fibroblast receptor affinities, compared with the intact peptides. The data demonstrate the importance of the C-terminal sequence for the enhancement of receptor binding and biological activity in fibroblasts. Further, it appears that the structural requirements (and thus the receptor) related to the acid inhibitory activity differ from those related to the mitogenic activity of these polypeptides.

INTRODUCTION

Epidermal growth factor–urogastrone (EGF-URO), a 53-residue single-chain polypeptide of about 6000 daltons, found in the mouse (1, 2) and in man (3–8), is both a potent stimulant of cell growth and an inhibitor of gastric acid secretion. A number of other biological actions of these peptides have been documented; there is also evidence to suggest the presence of similar polypeptides in a variety of species other than the mouse and man (summarized in Refs. 8 and 9). Specific membrane receptors for EGF-URO have been characterized in a variety of tissues from several species (10–13). Although the sequences of the mouse and human polypeptides differ at 16 positions (Fig. 1), previous work has demonstrated that both polypeptides can share the same receptor site in human fibroblasts (3, 14). The polypeptide of human origin has been termed urogastrone (URO) and that from the mouse has been termed epidermal growth factor (EGF), a distinction which will be retained for purposes of this communication. The term EGF-URO may be used to refer to the family of polypeptides that undoubtedly occurs in many species.

In the course of structural studies of the human polypeptide (5, 6), it was possible to obtain derivatives of both EGF and URO lacking up to six of the C-terminal

These studies, performed mainly in the Alan Bernstein Memorial Laboratories of the Johns Hopkins University School of Medicine, were supported in part by a grant from the National Foundation–March of Dimes (1-526).

[1] These data have been presented in part in preliminary form (16, 17).

0026-895X/80/030314-07$02.00/0

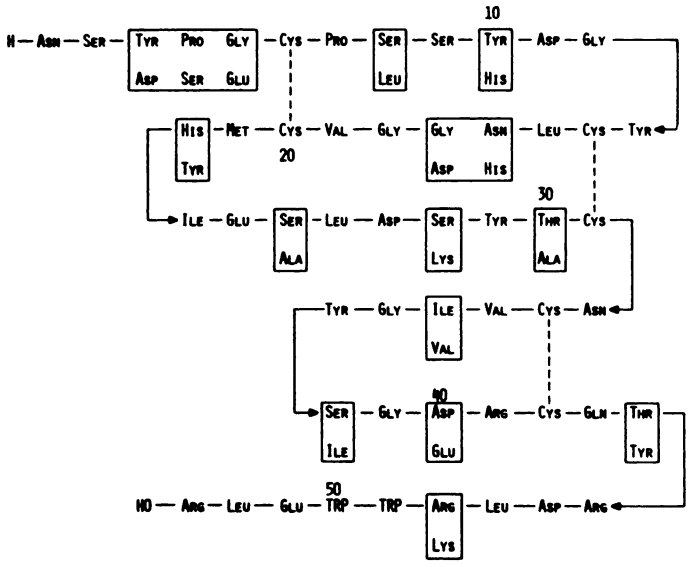

FIG. 1. *Amino acid sequence of mouse epidermal growth factor (upper sequence) and human urogastrone (lower sequence)*
The sequence differences are emphasized by the enclosed residues.

amino acids. The studies we describe here were done to evaluate the biological activities and receptor binding properties of these derivatives in comparison with observations with the intact polypeptides. The data amplify other studies performed with several derivatives of the mouse polypeptide (15) and extend our preliminary observations (16, 17).

MATERIALS AND METHODS

Studies with fibroblasts. Human fibroblast monolayer cultures (mycoplasma free), derived from normal adult male skin biopsies, were routinely propagated in 75-cm^2 plastic T flasks as previously described (10, 14) in antibiotic-free Eagle's minimal essential medium, with Earle's balanced salts, supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine. Cells were subcultured also as previously detailed (10, 12, 14) as 1.5-cm-diameter monolayers in 24-well multiwell trays (Linbro, New Haven, Conn.). After subculture from T flasks into multiwell trays, monolayers were refed twice at 3- to 4-day intervals with growth medium containing 5% (v/v) serum and were used 4 to 12 days after reaching confluency. Measurements of the binding of ^{125}I-labeled polypeptides and of the stimulation of [^{3}H-*methyl*]thymidine incorporation (1 μCi/ml; sp act, 6 Ci/mmol; Schwartz-Mann, Orangeburg, N.J.; counting efficiency, 13%) were performed in multiwell trays as recorded earlier (10, 12, 14) with minor modifications. Binding measurements were done at 24°C in Earle's buffer (18), pH 7.4, containing 25 mM Tris-HCl and 0.1% (w/v) crystalline bovine albumin (Pentex, 5× recrystallized, Calbiochem, La Jolla, Calif.). At this temperature, the degradation of ligand is minimized, and the affinities measured are in accord with those estimated in the presence of proteolysis inhibitors (10–13). Samples were allowed to equilibrate for 50 min, at which time cells were rinsed four times with ice-cold albumin-free buffer, solubilized for 10 min at 70°C in 0.1 N NaOH, and neutralized with 0.1 N HCl, and the radioactivity was measured by crystal scintillation counting (85% efficiency). The protein content of monolayers was determined with the Folin-Ciocalteu reagent (19). Where appropriate, the specific binding was calculated by subtracting from the net radioactive uptake, the amount bound in the presence of an excess (1 μg/ml) of unlabeled peptide (always less than 15% of the total binding). Measurements of peptide-stimulated thymidine incorporation were determined at 37°C over a 3-h period begun about 21 h after the addition of the peptide to the medium in which cells had reached confluency.

Preparation of peptides. Mouse EGF was prepared from frozen submaxillary glands (20). Intact human URO and the derivative lacking the C-terminal arginine residue (URO 1–52; also termed γ-urogastrone) were isolated from human urine (6). Derivatives of both URO and EGF lacking the six C-terminal amino acids (URO 1–47 or EGF 1–47) were prepared by limited enzymatic digestion of the intact peptides (5, 21, 22) followed by isolation of the cleaved peptides by gel filtration. EGF 1–47 was prepared by trypsin treatment of the intact peptide to yield EGF 1–48, isolated by Sephadex G-25 chromatography, followed by carboxypeptidase B treatment of EGF 1–48. The final product, EGF 1–47, was also isolated by Sephadex G-25 chromatography. URO 1–47 was prepared by digestion of the intact peptide with *Armillaria mellea* protease, followed by gel chromatography on Bio-Gel P6; this procedure also yielded the fragment URO 48–53. A synthetic sample of URO 48–52 was provided by Dr. J. Gormley of ICI. The peptide derivatives exhibited the expected changes in mobility upon analysis by acrylamide gel electrophoresis and yielded the correct compositions when subjected to amino acid analysis. The concentrations of peptides, redissolved for study after lyophilization, were determined from the absorption at 215 and 225 nm, according to the formula: $(E_{215}^{1\,cm} - E_{225}^{1\,cm}) \times 155 = \mu g/ml$ (23).

Measurement of inhibition of acid secretion. The potencies of samples were determined in dogs with denervated fundic (Heindenhain) pouches as previously described (24, 25). Briefly, gastric acid secretion was stimulated by a continuous infusion of histamine to give a plateau at 50–70% of the maximum output in each dog. The output was determined by collecting secretion at 15-min intervals to measure volume and also total acid. Test samples were given by iv injection and their potencies expressed as the percentage by which the plateau volume is reduced at maximum inhibition—usually observed in the second sample following administration. The amount of inhibitor of the urogastrone type which is required is dependent upon the level of secretion evoked and varies from dog to dog. In any one day, two active samples can be tested in a given dog; dogs can only be used on alternate days.

RESULTS

Biological activity in fibroblasts. In keeping with previous observations (8, 12–14), all of the peptides examined were potent stimulants of thymidine incorporation in human fibroblast monolayers (Figs. 2–3). However, the intact polypeptides (URO 1–53 and EGF 1–53) and the human peptide lacking the C-terminal arginine (URO 1–

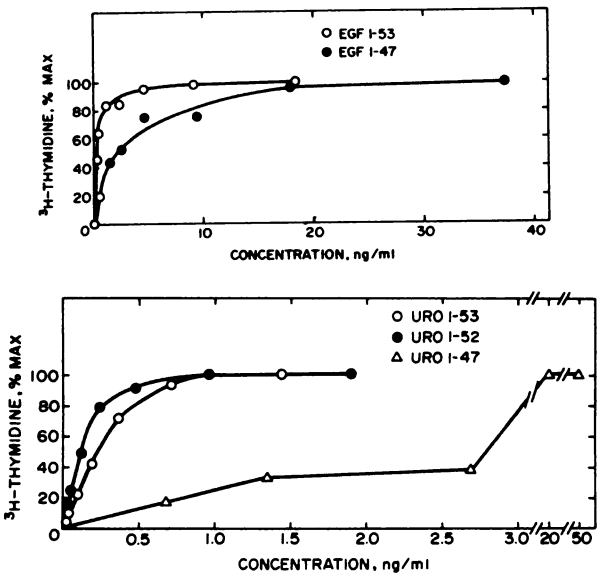

FIG. 2. *Dose-response curves for stimulation of human fibroblast thymidine incorporation by derivatives of urogastrone and epidermal growth factor*

Response curves for each peptide were determined in separate 24-well multidish trays by the addition of increasing amounts of peptide to the depleted growth medium in which monolayers had reached confluency. The incorporation of ^{3}H-thymidine (1 μCi/ml) into trichloroacetic acid-insoluble material was measured during a 3-h period begun 22 h after the addition of peptide. Values representing the average of triplicate measurements that varied less than 10% are expressed as a percentage of the maximum incorporation above baseline; when compared simultaneously in a single monolayer tray, all peptides at high concentrations caused the same maximal response.
Upper: Response curve for intact epidermal growth factor (EGF 1–53; ○) and the derivative lacking six C-terminal amino acids (EGF 1–47; ●).
Lower: Response curve for intact urogastrone (URO 1–53; ○) and the derivatives lacking either one (URO 1–52; ●) or six (URO 1–47; △) C-terminal amino acids.

52 or γ-urogastrone) were about 10-fold more potent than the derivatives lacking the six C-terminal amino acids (URO 1–47 or EGF 1–47; Fig. 2 and Table 1). URO 1–47 and EGF 1–47 are approximately equipotent (Fig. 3 and Table 1). Importantly, all derivatives at comparatively high concentrations are capable of causing the same

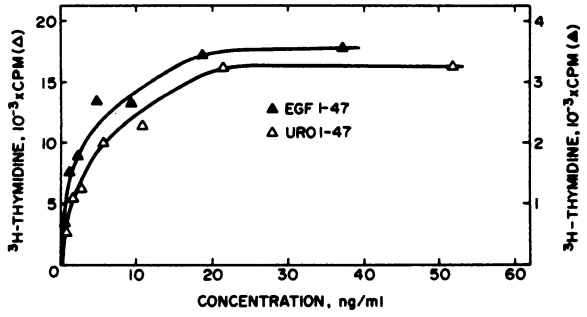

FIG. 3. *Comparative dose-response curves for derivatives of epidermal growth factor and urogastrone lacking six C-terminal amino acids*

Dose-response curves for EGF 1–47 (▲) and URO 1–47 (△) were determined in separate monolayer trays as outlined in the legend to Fig. 2. The data are corrected for the incorporation measured in the absence of peptide (2960 cpm for URO 1–47; 140 cpm for EGF 1–47).

TABLE 1

Comparison of receptor binding and biological activities of derivatives of EGF-URO

The binding and biological activities were determined both for intact human and mouse EGF-URO (URO 1–53; EGF 1–53) and for derivatives lacking either one (URO 1–52) or six (URO 1–47; EGF 1–47) carboxy-terminal amino acids. The dissociation constants and ED_{50}'s for ^{3}H-thymidine incorporation were determined in human fibroblast monolayers as illustrated in Figs. 2, 7 and 8. The acid inhibitory potencies determined *in vivo* are representative of values obtained in one experimental series. Values represent the mean ± ½ range of two observations or the mean ± SD for the number of estimates indicated in parentheses.

Compound	Binding K_D	Bioactivity	
		^{3}H-Thymidine ED_{50}	Acid inhibition ED_{50}
	ng/ml	*ng/ml*	*μg/kg*
URO 1–53	0.57 ± 0.11 (5)	0.27 ± 0.05 (5)	0.5–0.6
URO 1–52	0.48 ± 0.10 (3)	0.13 ± 0 (2)	0.5–0.6
URO 1–47	6.3 ± 2.0 (4)	3.2 ± 0.7 (2)	0.5–0.6
EGF 1–53	1.6 ± 0.4 (4)	0.24 ± 0.11 (10)	0.5–0.6
EGF 1–47	7.2 ± 2.3 (6)	2.1 ± 0.5 (3)	0.5–0.6

maximal biological response. The C-terminal hexapeptide, URO 48–53, was inactive on its own and did not affect the potency of the shortened peptide, EGF 1–47 (data not shown). Synthetic URO 48–52 was similarly inactive. As previously noted (10, 12, 14), the shapes of the dose-response curves (as illustrated in Figs. 2 and 3) were highly reproducible, irrespective of either the relative or the absolute stimulation of thymidine incorporation observed; reproducible values of the ED_{50} were thus obtained from such dose-response curves (Table 1).

Inhibition of acid secretion. Precise assays of stimulants of gastric acid secretion are difficult to achieve. Furthermore, since inhibitors are measured against such a stimulant, an additional set of variables is introduced. Thus, given the inherent difficulties of the bioassay and the limited amounts of peptide derivatives available for studies *in vivo*, it was not possible to obtain antisecretory data with anything like the precision of the thymidine incorporation assay. Urogastrone-mediated inhibition of acid secretion gives a sharp dose-response curve as shown for an individual dog (Fig. 4). Thus, samples giving a low level of inhibition (e.g., 30–40%) give a high level merely by doubling the dose. The potencies of test samples were determined using single doses on different days, but using the same dogs throughout. For comparison of different samples in an individual animal, the potency can be expressed as the dose causing 50–70% inhibition. Unfortunately, this bioassay does not permit a meaningful statistical estimate of the interassay experimental error. Nonetheless, despite these drawbacks of the assay, the use of this method over a prolonged period (6) showed that twofold differences in potency were clearly observable. Thus, although only representative values of potency can be given (Table 1), within the limits of this test system, the abilities of URO 1–53, URO 1–52, URO 1–47, EGF 1–53, and EGF 1–47 to inhibit gastric secretion were indistinguishable.

Evaluation of methods for measuring the binding affinity of unlabeled polypeptides in fibroblast mono-

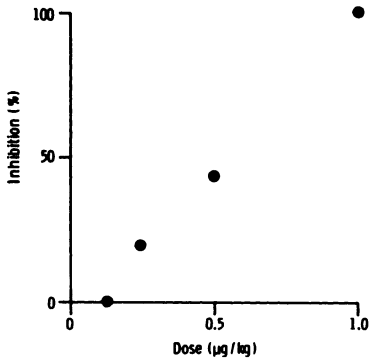

FIG. 4. *Dose-response curve for inhibition of histamine-mediated gastric acid secretion*

A representative experiment is depicted for the inhibitory action of single intravenous doses administered to a dog on different days. The actions of URO 1–53, URO 1–47, and EGF 1–47 are indistinguishable in this assay.

layers. In previous work (3, 10–14), the receptor binding affinity of ^{125}I-labeled EGF or URO has been measured. The insertion of iodine into EGF is known not to alter the biological activity appreciably (13, 26), and the same may be assumed to hold for URO. However, the locations of the tyrosine residues, at which sites iodine may be incorporated, differ considerably between EGF and URO (Fig. 1). Thus, for comparative purposes, it was thought to be an advantage to measure the receptor affinity of the unlabeled polypeptides. Given the limited amounts of peptides available for these studies and the limited

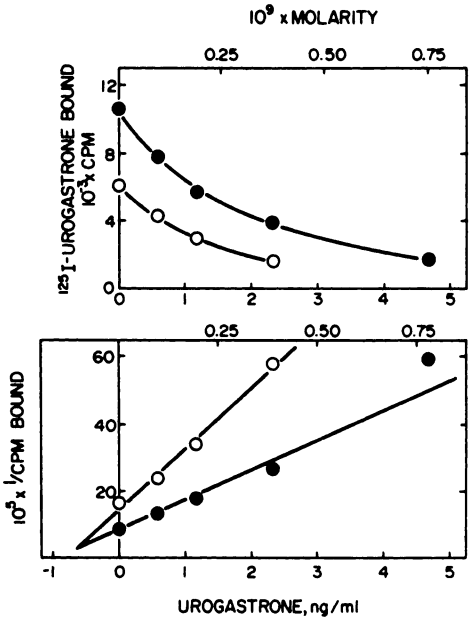

FIG. 5. *Estimate of the affinity of unlabeled urogastrone: Dixon plot of binding inhibition data*

Confluent fibroblast monolayers (41 μg cell protein per monolayer) in a 24-well multidish tray were incubated at 24°C with two concentrations of ^{125}I-labeled urogastrone (539 cpm/pg; ●, 1.9 ng/ml, and 0, 0.95 ng/ml) in the absence and presence of increasing concentrations of unlabeled urogastrone. The specific binding of radioactive urogastrone was determined and the binding-competition data (upper) were used to calculate the Dixon plot (lower) so as to yield from the intersection point, a value for the dissociation constant of unlabeled urogastrone.

TABLE 2

Comparison of binding affinities of unlabeled EGF and URO determined by several methods

The dissociation constants for native EGF and URO were estimated as detailed in the text from data like those depicted in Figs. 7 and 8 (binding competition), Figs. 5 and 8 (Dixon plot), and Fig. 6 (dose ratio). Values represent the mean ± ½ range for two estimates or the mean ± SD for the number of estimates indicated in parentheses.

Compound	Method of assay	K_i
		ng/ml
EGF	Binding competition	1.6 ± 0.4 (4)
EGF	Dixon plot	1.7 ± 0.2 (2)
EGF	Dose ratio	1.0 ± 0.3 (3)
URO	Binding competition	0.57 ± 0.11 (5)
URO	Dixon plot	0.63 ± 0.09 (3)
URO	Dose ratio	0.34 ± 0.14 (3)

number of tissue samples in a 24-well multiwell tray, it was necessary to determine the most economic and reliable measure of unlabeled peptide affinity.

Determinations of binding-competition curves (e.g., Fig. 5, upper) yield the most precise data with the largest number of replicate data points. A single curve (Fig. 5, upper), yielding the concentration of unlabeled polypeptide (IC_{50}) at which the binding of radiolabeled peptide is 50% of maximum, can be used to determine the dissociation constant of unlabeled peptide (K_i) according to the equation (27):

$$K_i = (IC_{50})/[1 + (L^*/K_D^*)].$$

For this estimate, it is necessary to know accurately both the dissociation constant of the labeled peptide (K_D^*) and the concentration of labeled peptide (L^*). Fortunately, the measured K_D^*'s for ^{125}I-labeled EGF and URO have been quite reproducible in this laboratory, both for a number of different preparations of ^{125}I-labeled peptides and for a variety of human fibroblast samples: $K^*_{D,\,EGF}$ = 4.3 ± 0.7 ng/ml (N = 10); $K^*_{D,\,URO}$ = 1.4 ± 0.2 ng/ml (N = 5). A reliable calculation of the affinity of the unlabeled peptide was thus possible (Tables 1 and 2), based on the previous formula.

Two other approaches were used to estimate the receptor affinity of unlabeled URO and EGF. First, data from two binding-competition curves, measured in the same multiwell tray at two concentrations of ^{125}I-labeled peptide were analyzed according to the equations for enzyme inhibition (e.g., see Ref. 28); the unlabeled peptide is considered the "inhibitor" for binding of the labeled peptide "substrate." A Dixon-type analysis yields, from the intersection point, the affinity of the unlabeled polypeptide (Fig. 5, lower). Second, a variant of the "dose-ratio" method (29) was used, whereby the binding of ^{125}I-labeled polypeptide at increasing concentrations was measured in both the absence and the presence of several concentrations of unlabeled peptide. The increased concentration of ^{125}I-labeled peptide, yielding the same amount of radioligand bound as that observed in the absence of competitor, is used to calculate the dose ratio and hence the binding constant for unlabeled peptide, as outlined in Fig. 6 (29). Neither the affinity of the labeled peptide nor the absolute concentration of labeled peptide need be known accurately for this estimate.

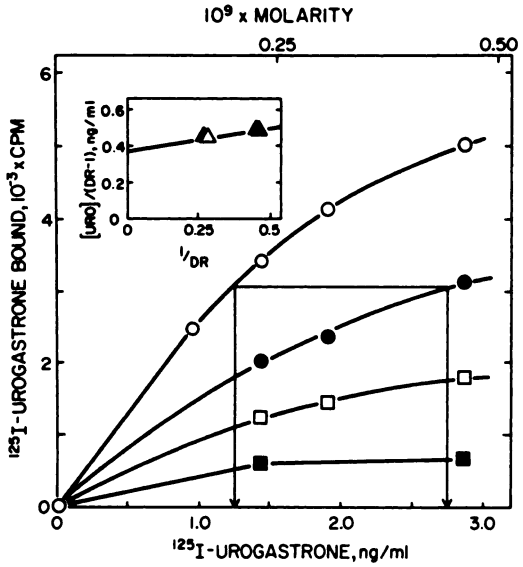

FIG. 6. *Estimate of the affinity of unlabeled urogastrone by the dose-ratio method*

The specific binding at increasing concentrations of ^{125}I-labeled urogastrone (378 cpm/pg) to fibroblast monolayers (29 μg cell protein per monolayer) was determined in the absence (○) and presence of three fixed concentrations of unlabeled urogastrone: ●, 0.56 ng/ml; □, 1.12 ng/ml; and ■, 2.24 ng/ml. From the three upper binding isotherms, dose ratios (DR) were estimated, as indicated by the arrows, and a plot of [urogastrone]/(DR-1) versus 1/DR was constructed as shown in the inset, to yield as the *Y* intercept, the dissociation constant for unlabeled urogastrone (29). The inset shows data calculated from two independent experiments.

Unfortunately, the small sample size of the multiwell tray and the inability to achieve sufficiently high concentrations of radiolabeled peptide severely limit the usefulness of this approach. For instance, at the highest concentration of unlabeled URO, it was not possible to raise the concentration of ^{125}I-labeled URO sufficiently to measure an accurate dose ratio (Fig. 6). Nonetheless, all three methods yielded values for the K_i's of unlabeled URO and EGF that were in quite good agreement (Table 2). The binding-competition method provided the largest number of replicate data points with the best interassay reproducibility; this method, using a single competition curve (e.g., Fig. 7), was, therefore, chosen to study the other polypeptides.

Binding of derivatives of URO and EGF to fibroblasts. In accord with the measurements of biological potency in fibroblasts, the intact polypeptides (URO 1–53 and EGF 1–53) possessed much higher binding affinities than did the derivatives lacking the C-terminal hexapeptide (Figs. 7 and 8; Table 1). Similarly, URO 1–52 (or γ-urogastrone), which is equipotent with URO, in the fibroblast bioassay possessed an affinity equal to that of the intact peptide (Table 1), while the biologically inactive C-terminal hexapeptides (URO 48–53 and URO 48–52) were devoid of receptor binding activity (data not shown); reduced, carboxamidated URO 1–52 was also inactive.

DISCUSSION

It is remarkable that despite 16 differences between the amino acid sequences of human URO and mouse EGF, both polypeptides are equipotent in the fibroblast and dog bioassays, and both bind to the human fibroblast receptor with similar affinities. The binding studies that we report here in greater detail than previously described suggest a slightly higher receptor binding affinity for the unlabeled polypeptides compared with the iodinated polypeptides.

The data for the binding affinity of both labeled and unlabeled EGF and URO also indicate a two- to threefold higher binding affinity of the human polypeptides for the human fibroblast receptor. Such an increased affinity may have been anticipated. It is more striking, however, that the affinities of EGF and URO are so similar, rather than that they differ slightly, given the many sequence differences distributed widely throughout the molecules as noted previously. It may be of significance that in the first two interconnected loops of the molecule (residues 1–31), the amino acid substitutions (e.g., mouse → human) comprise changes (e.g., neutral → basic or acidic residues) that would markedly alter the chemical microenvironment of the two peptides. In contrast, the differences between EGF and URO in the sequence 33–53, comprising the final "loop and tail" of the peptide, represent chemically "conservative" changes in the molecule (e.g., Ile^{35} → Val ^{35}; Asp^{40} → Glu^{40}; Arg^{48} → Lys^{48}). This relative sequence conservation between EGF and URO in the sequence 33–53 may point to its importance in the receptor interaction so as to account for the comparable binding affinities of EGF and URO.

The studies with the derivatives that we report here, along with other data that appeared during the course of our work (15), have focused principally on the C-terminal portion of the molecule. In accord with Cohen and collaborators (15), who observed that EGF 1–51 is equipotent with intact EGF, both in receptor binding and in the human fibroblast thymidine incorporation assay, we ob-

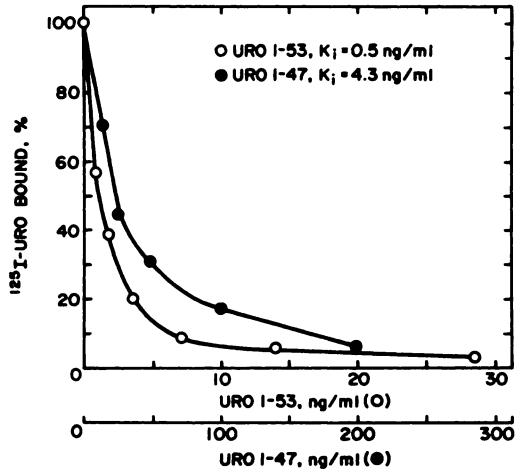

FIG. 7. *Binding of URO 1–53 and URO 1–47 to fibroblast monolayers*

The competition for the binding of ^{125}I-labeled urogastrone (○, 1.44 ng/ml; ●, 5.74 ng/ml) by intact urogastrone (URO 1–53; ○) and by the shortened peptide (URO 1–47; ●) was measured for increasing concentrations of unlabeled peptide. The unlabeled peptide concentration for which the binding of labeled ligand was reduced to 50% of the amount bound in the absence of competitor (IC_{50}) was used to calculate the inhibitor dissociation constant according to the equation (27): $K_i = IC_{50}/(1 + L^*/K^*)$, where L^* and K^* represent, respectively, the concentration and dissociation constant of ^{125}I-labeled urogastrone.

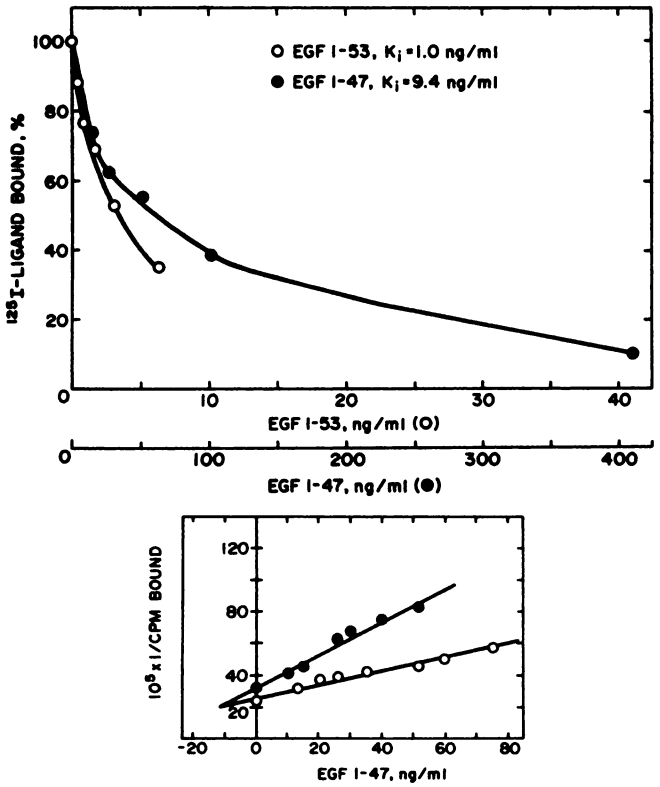

FIG. 8. *Estimate of the fibroblast affinity of unlabeled EGF 1–53 and EGF 1–47*

Upper: Binding-competition curves and peptide affinities were determined as outlined in Fig. 7 for EGF 1–53 (O) and EGF 1–47 (●) using either ^{125}I-labeled epidermal growth factor (17.1 ng/ml; ●) or ^{125}I-labeled urogastrone (1.9 ng/ml; O).

Lower: The affinity of unlabeled EGF 1–47 was determined in the presence of ^{125}I-labeled epidermal growth factor using a Dixon plot as outlined in Fig. 5.

serve that URO 1–52 is equipotent with intact URO. In contrast, there is a marked reduction in fibroblast receptor affinity and in the fibroblast biological potency of derivatives (EGF 1–47; URO 1–47) lacking the six C-terminal amino acids. Nonetheless, these derivatives can cause the same maximum biological responses in fibroblasts as do the intact peptides, which are about 10-fold more potent. Our results can be compared with those of Cohen *et al.* (15), who observed that at 50 ng/ml, EGF 1–48 caused about the same stimulation of fibroblasts as did 0.5 ng/ml EGF; EGF 1–48 was also reported to be 5% as effective as the native molecule in competing for the fibroblast binding of ^{125}I-labeled EGF (15). Because of the enzymatic methods used to prepare the derivatives, alterations in the molecule other than the loss of the C-terminal portion cannot be entirely ruled out. For instance, trypsin might also cleave EGF at residues 41–42 and 45–46 and the armillaria mellea protease might open the interconnected loops comprising residues 14–31. However, when URO 1–47 was reduced and reoxidized, biological activity was regained; thus little significant cleavage at Lys 28 of URO appears to have occurred. Further, the similarity in the reduction in receptor binding and fibroblast potencies for the derivatives of EGF and URO, prepared by enzymatically dissimilar methods, argues in favor of the changes being attributable solely to the loss of the terminal hexapeptide.

On the whole, the reduced biological potencies of the derivatives in fibroblasts are paralleled by a concomitant reduction in fibroblast receptor affinity. Taken together, our data and those of Cohen *et al.* (15) indicate the importance of the C-terminal sequence 49–51 (Trp, Trp, Glu) for the enhancement of receptor binding and consequently the biological activity in fibroblasts. Significantly, the terminal hexapeptide (URO 48–53) alone is inactive in fibroblasts, does not compete for binding at the receptor site, and does not possess sufficient affinity for the complementary N-terminal sequence 1–47 to enhance the potency of EGF 1–47 in the fibroblast assay. Thus, although the terminal hexapeptide is important for receptor binding, the intrinsic biological activity of the molecule resides elsewhere in the sequence.

A somewhat surprising finding of this study concerns the equal biological activities of intact URO and URO 1–47 in the acid inhibition bioassay *in vivo*, as opposed to the 10-fold lower potency of URO 1–47, compared with URO 1–53, both in receptor binding and in the fibroblast thymidine incorporation assay *in vitro*. It is possible that, *in vivo*, URO 1–53 is rapidly cleaved to URO 1–47, such that the activity of the intact peptide is not truly measured. This possibility has been suggested to explain the apparently equal activities of EGF 1–48 and intact EGF when assayed over the relatively long-term period of the mouse bioassay *in vivo* (15, 30). However, it is unlikely that this possibility explains the equal potency of URO 1–53 and URO 1–47 in the acid inhibition bioassay, which monitors a rapid response to either an intravenous bolus or a constant infusion of peptide; the urinary excretion of large amounts of URO and URO 1–52 and the lack of evidence for significant amounts of URO 1–47 in urine (4–6) argue against a degradative mechanism to explain the equal potencies of URO 1–47 and URO 1–53. An attractive hypothesis to explain the different relative potencies of URO 1–53 and URO 1–47 (*in vivo* versus *in vitro*) is that the receptor involved in the inhibition of acid secretion may differ from the one involved in fibroblast stimulation. It will thus be of considerable interest to examine other analogues of EGF-URO, particularly those modified in the sequence 33–42, to determine if the two principal intrinsic biological activities of the molecule can be selectively altered.

ACKNOWLEDGMENT

The incomparable technical assistance of W. H. Shackelford is gratefully acknowledged.

REFERENCES

1. Cohen, S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J. Biol. Chem.* **237**: 1555–1562 (1962).
2. Cohen, S. Epidermal growth factor. *J. Invest. Dermatol.* **59**: 13–16 (1972).
3. Cohen, S. and G. Carpenter. Human epidermal growth factor: Isolation and chemical and biological properties. *Proc. Natl. Acad. Sci. U.S.A.* **72**: 1317–1321 (1975).
4. Gregory, H. Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature (London)* **257**: 325–327 (1975).
5. Gregory, H. and B. M. Preston. The primary structure of human urogastrone. *Int. J. Peptide Protein Res.* **9**: 107–118 (1977).
6. Gregory, H. and I. R. Willshire. The isolation of the urogastrones—inhibitors of gastric acid secretion from human urine. *Hoppe-Seylers Z. Physiol. Chem.* **356**: 1765–1774 (1975).
7. Carpenter, G. The regulation of cell proliferation: Advances in the biology of action of epidermal growth factor. *J. Invest. Dermatol.* **71**: 283–288 (1978).
8. Hollenberg, M. D. Epidermal growth factor-urogastrone: A polypeptide acquiring hormonal status. *Vitamins Hormones* **37**: 69–110 (1979).

9. Gregory, H., J. M. Bower and I. R. Willshire. Urogastrone and epidermal growth factor, in *Growth Factors* (K. W. Kastrup and J. H. Nielsen, eds.). FEBS Colloquium B3. Pergamon Press, New York, Vol. 48, 75–84 (1972).
10. Hollenberg, M. D. and P. Cuatrecasas. Epidermal growth factor. Receptors in human fibroblasts and modulation of action by cholera toxin. *Proc. Natl. Acad. Sci. U. S. A.* **70:** 2964–2968 (1973).
11. O'Keefe, E., M. D. Hollenberg and P. Cuatrecasas. Epidermal growth factor. Characteristics of specific binding in membranes from liver, placenta and other target tissues. *Arch. Biochem. Biophys.* **164:** 518–526 (1974).
12. Hollenberg, M. D. and P. Cuatrecasas. Insulin and epidermal growth factor: Human fibroblast receptors related to deoxyribonucleic acid synthesis and amino acid uptake. *J. Biol. Chem.* **250:** 3845–3853 (1975).
13. Carpenter, G., K. J. Lembach, M. M. Morrison and S. Cohen. Characterization of the binding of ^{125}I-labeled epidermal growth factor to human fibroblasts. *J. Biol. Chem.* **250:** 4297–4303 (1975).
14. Hollenberg, M. D. and H. Gregory. Human urogastrone and mouse epidermal growth factor share a common receptor site in cultured human fibroblasts. *Life Sci.* **20:** 267–274 (1977).
15. Cohen, S., G. Carpenter and K. J. Lembach. Interaction of epidermal growth factor (EGF) with cultured fibroblasts. *Adv. Metab. Disord.* **8:** 265–284 (1975).
16. Hollenberg, M. D. and H. Gregory. Urogastrone-EGF derivatives: Differential effects on acid secretion and thymidine incorporation. *Clin. Res.* **25:** 312A (1977).
17. Hollenberg, M. D. and H. Gregory. Urogastrone-EGF: Receptor binding and activities of derivatives. Abstracts, 2nd Congress, Pan American Association of Biochemical Societies, Caracas, Venezuela, 27 (1978).
18. Earle, W. R. Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. *J. Natl. Cancer Inst.* **4:** 165–212 (1943).
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275 (1951).
20. Savage, C. R., Jr. and S. Cohen. Epidermal growth factor and a new derivative. Rapid isolation procedures and biological and chemical characterization. *J. Biol. Chem.* **247:** 7609–7611 (1972).
21. Walton, P. L., R. W. Turner and D. Broadbent. U.K. Patent Specification No. 1263956 (1972).
22. Gregory, H. The preparation of desIysylalanyl porcine and bovine insulin. *FEBS Lett.* **51:** 201–205 (1975).
23. Hollenberg, M. D. and D. B. Hope. The composition of crystalline complexes of neurophysin-M with (8-arginine)-vasopressin and oxytolin. *Biochem. J.* **105:** 921–926 (1967).
24. Gerring, E. L. and E. Hawarth. The effects of urogastrone on gastric acid secretion in dogs and cats. *Rend. Rom. Gastroenterol.* **2:** 167–171 (1970).
25. Gerring, E. L., J. M. Bower and H. Gregory. Urogastrone: A potential drug in the treatment of duodenal ulcer, in *Chronic Duodenal Ulcer* (C. Wastell, ed.). Butterworths, London, 171–180 (1974).
26. Hollenberg, M. D. Receptors for insulin and epidermal growth factor: Relationship to synthesis of DNA in cultured rabbit lens epithelium. *Arch. Biochem. Biophys.* **171:** 371–377 (1975).
27. Cheng, Y.-C. and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition of an enzymatic reaction. *Biochem. Pharmacol.* **22:** 3099–3108 (1973).
28. Segel, I. H. *Enzyme Kinetics*. John Wiley and Sons, New York (1975).
29. Chang, K.-J., S. Jacobs and P. Cuatrecasas. Quantitative aspects of hormone-receptor interactions of high affinity: Effect of receptor concentration and measurement of dissociation constants of labeled and unlabeled hormones. *Biochim. Biophys. Acta* **406:** 294–303 (1975).
30. Savage, C. R., Jr., T. Inagami and S. Cohen. The primary structure of epidermal growth factor. *J. Biol. Chem.* **247:** 7612–7621 (1972).

Send reprint requests to: Morley D. Hollenberg, Division of Pharmacology and Therapeutics, University of Calgary Faculty of Medicine, Calgary, Alberta, Canada T2N 1N4.