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Two forms of murine epidermal growth factor: Rapid separation by using reverse-phase HPLC

(rapid HPLC purification/iodination/radioimmunoassay)

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ABSTRACT **Epidermal growth factor (EGF) has been isolated from acid extracts of C57BL6/J mouse submaxillary glands by using hydrophobic chromatography. High yields of EGF in large amounts (10 mg) can be isolated reliably from the acid extract of the glands in less than 4 hr. The reverse-phase HPLC techniques used to purify the EGF initially yielded what appeared to be a single homogeneous EGF molecule. However, ion pairing reagents (e.g., heptafluorobutyric acid) altered the chromatographic properties, revealing two distinct species: EGF-α and EGF-β. The apparent molecular weights, isoelectric points, and antigenic properties of EGF-α and EGF-β were identical, and both forms stimulated a mitogenic response in 3T3 cells. Analysis of different preparations of purified EGF (commercial and experimental) indicated the presence of EGF-α and EGF-β in constant proportion. Previous EGF binding studies must have used mixtures of ^{125}I-labeled EGF-α and ^{125}I-labeled EGF-β. The two molecules appear to compete for an identical receptor on the cell surface.**

Epidermal growth factor (EGF) can be isolated from the submaxillary glands of mice (1) by using gel filtration and ion exchange chromatography (2). This procedure relies on a weak interaction between EGF and the gel filtration matrix and results in considerable dilution of the EGF (2). To detect the EGF, it is necessary to perform radioimmunoassays. In this report, we describe procedures for the trace enrichment (3–5) of EGF from an extract of mouse salivary glands and the subsequent reverse-phase HPLC (RP-HLPC), which yield highly purified EGF in three rapid steps. During this work it became possible to separate EGF into two closely related proteins, EGF-α and EGF-β. There is an extensive literature on the interaction of EGF with its target cells (6) and on the structure–function relationships for EGF (7), which will now have to be reevaluated because the EGF prepared by previous procedures contained both EGF-α and EGF-β.

MATERIALS AND METHODS

Preparation of EGF by Using RP-HLPC. *Preparative trace enrichment.* Male C57BL6/J mice were killed by cervical dislocation, and the submaxillary glands were removed and flash frozen in liquid nitrogen. Frozen glands (20 g) were homogenized by using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Federal Republic of Germany) in 10 vol of ice-cold extraction buffer [1% CF_3COOH/5% HCOOH (vol/vol)/1% NaCl] in 1 M HCl. The homogenate was clarified by centrifugation at 175,000 × *g* at 4°C for 30 min and the resulting supernatant fluid was defatted by suction filtration through nylon wool. The acidic supernatant was made 20% (vol/vol) with respect to acetonitrile and loaded directly onto a 9 cm × 1 cm glass column packed with Waters preparative C_{18} (55–105 μm) reverse-phase material (Waters Associates, part 51922) that had previously been equilibrated with 20% acetonitrile/80% H_2O/0.1% CF_3COOH. The column was washed with 3.5 vol of equilibration buffer and an EGF-enriched fraction was recovered by elution (flow rate, 3 ml/min) with 3.5 vol of 50% acetonitrile/50% H_2O/0.1% CF_3COOH.

HPLC. All separations were carried out at ambient temperature and a constant flow rate of 1 ml/min. Operating parameters were controlled by using an Altex model 324-40 chromatograph (Altex Scientific, Berkeley, CA) fitted with a 2-ml sample injection loop. Eluted proteins were detected by using a LDC Spectromonitor III variable-wavelength spectrophotometer (Laboratory Data Control, City, Florida). The trace-enriched fraction was diluted 1:1 with distilled water to decrease the acetonitrile concentration sufficiently to allow hydrophobic interaction and loaded in multiple 2-ml aliquots onto a 15 cm × 4.6 mm inside diameter (i.d.) stainless steel column packed with Ultrasphere-ODS (Altex Scientific) equilibrated with a primary solvent of 0.2% heptafluorobutyric acid (C_3F_7COOH)/H_2O, pH 2.3. After loading, the chromatogram was developed by running a linear 50-min gradient between this primary solvent and a secondary solvent of 50% acetonitrile/50% H_2O/0.2% C_3F_7COOH. Material eluting between 36 and 40 min was recovered and rechromatographed on a 30 cm × 9 mm (i.d.) C_{18} semipreparative column (Waters Associates, part 84176) with a 50-min gradient between 32.5% acetonitrile/67.5% H_2O/0.2% C_3H_7COOH and 40% acetonitrile/60% H_2O/0.2% C_3H_7COOH. On this system, EGF-α eluted after a retention time of 26.6 min and EGF-β eluted after 28.7 min. These EGF materials were further purified from contaminating proteins of similar relative hydrophobicity by using a 30 × 7.5 mm (i.d.) TSK3000SW size-exclusion column (Altex Scientific) equilibrated with aqueous 0.1 M phosphate (pH 6.5).

Characterization and analysis of the various stages of the EGF preparation were carried out on the 15-cm Ultrasphere-ODS column by using the gradient elution conditions defined for the preparative separation. Under these conditions, EGF-α and EGF-β reproducibly chromatographed with retention times of 45 and 46 min, respectively.

Iodination of EGF. This was carried out by using chloramine-T as described (8) except that no carrier bovine serum albumin was added. The ^{125}I-labeled EGF was separated from free ^{125}I by RP-HPLC using disposable C_{18} SepPak cartridges (Waters

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Abbreviations: EGF, epidermal growth factor; RP-HLPC, reverse-phase HPLC; i.d., inside diameter.

Associates, part 51910) according to the following procedure: cartridges were washed with 5 ml of 60% acetonitrile/40% H_2O/0.2% C_3F_7COOH and then equilibrated with 10 ml of aqueous 0.2% C_3F_7COOH. The chloramine-T reaction mixture, after the addition of sodium metabisulfite, was diluted with 4 ml of aqueous 0.2% C_3F_7COOH and loaded onto the Sep-Pak cartridge. Under these conditions, free ^{125}I did not bind. The column was washed with 10 ml of 0.2% C_3F_7COOH and ^{125}I-labeled EGF was recovered from the column with three 1-ml portions of 50% acetonitrile/50% H_2O/0.2% C_3F_7COOH.

EGF Assays. EGF was assayed by using newborn mice (1), mouse 3T3 fibroblasts (9), solid-phase radioimmunoassay (10), and gel immunodiffusion (11).

Electrophoretic Analysis. The EGF preparations were analyzed by the method of Laemmli (12) using 15% polyacrylamide gels. Analytical isoelectric focusing was carried out on thin-layer agarose gels (13) using Pharmalyte 4–6.5. The gels were fixed in 10% (wt/vol) aqueous sulfosalicylic acid and 5% (wt/vol) CCl_3COOH for 1 hr and directly stained using Coomassie brilliant blue G250 in 5% (vol/vol) perchloric acid.

Amino Acid and Peptide Analysis. Amino acid analyses of EGF-α and EGF-β were carried out as follows: 30–50 μg of protein was hydrolyzed at reduced pressure at 108°C for 24 hr in 0.5 ml of constant boiling HCl containing phenol. For tryptophan determination, proteins were hydrolyzed for 24 hr in methanesulfonic acid containing tryptamine (2 mg/ml) (14). Hydrolysates were analyzed on a modified Beckman amino acid analyzer. End groups were determined as dansyl derivatives and identified on polyamide sheets (15, 16).

RESULTS

Reverse-Phase Chromatographic Properties of EGF. To determine the optimum chromatographic conditions for purification of EGF by gradient elution RP-HPLC, material purified by the two-step procedure of Savage and Cohen (2) was chromatographed on an Ultrasphere ODS column with three different solvent systems: 0.1 M sodium phosphate (pH 2.1), 0.2% CF_3COOH, and 0.2% C_3F_7COOH (Fig. 1). In the sodium phosphate buffer, EGF eluted as a single symmetrical peak (Fig. 1*A*). However, when the ion-pairing solvent CF_3COOH with an acetonitrile gradient of 1%/min was used, evidence of a second component was obtained (Fig. 1*B*) and a shallower gradient of acetonitrile gave partial resolution into two peaks. These two peaks, EGF-α and EGF-β, could be well resolved by using an acetonitrile gradient of 1%/min in C_3F_7COOH buffer instead of CF_3COOH (Fig. 1*C*), when EGF-α eluted at 45 min and EGF-β eluted at 46 min.

We have compared the chromatographic properties of EGF prepared by T. A. A. Dopheide (Commonwealth Scientific and Industrial Organization) (2), EGF purchased from Collaborative Research (Waltham, MA), and EGF prepared in our own laboratory. The major protein peaks in all of these preparations eluted from the Ultrasphere ODS column with the same retention times as EGF-α and EGF-β (Figs. 1 and 2). In all cases, the relative proportions of EGF-α and EGF-β were similar (≈4:1).

An analytical RP-HPLC protein profile (≈400 μg) of trace-enriched EGF and the corresponding EGF mitogenic activity and EGF radioimmunoassay profiles is shown in Fig. 2. The bioassay profile and radioimmunoassay data (Fig. 2*B*) indicated that both the EGF mitogenic and the EGF antigenic activities were associated with the EGF protein peaks (Fig. 2*A*).

Preparation of EGF-α and EGF-β. The recovery of EGF from the salivary gland extract by chromatography on the Waters C_{18} silica column was 100%. It was possible to monitor the sequential stages of purification of EGF from the original supernatant by using analytical RP-HPLC on the Ultrasphere ODS (C_{18}) columns. The optical density at 280 nm was integrated to quantitate the EGF content. A single extraction yielded only 40% of the EGF present in the glands, and it was necessary to repeat the homogenization twice to extract 90%

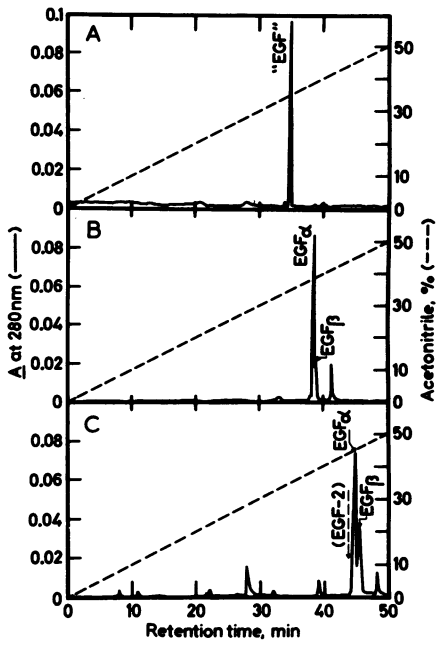

FIG. 1. Comparison of solvent systems for RP-HPLC separation of EGF. Proteins were separated on 15 cm × 4.6 mm (i.d.) columns of Ultrasphere ODS at ambient temperature and a flow rate of 1 ml/min. Gradient elution was between a primary solvent of 0.1 M sodium phosphate (pH 2.1) (*A*), 0.2% CF_3COOH (*B*), or 0.2% C_3F_7COOH (*C*) and acetonitrile (---). Detection was by UV absorption at 280 nm.

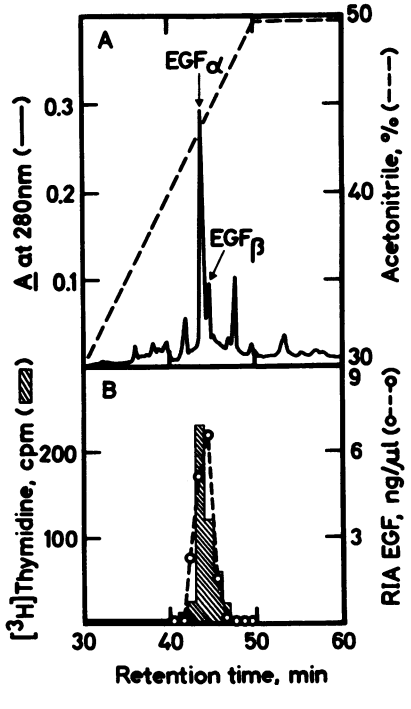

FIG. 2. Analytical separation of trace-enriched EGF from an acid extract of mouse submaxillary glands by chromatography on Ultrasphere ODS. (*A*) Protein profile and acetonitrile gradient. (*B*) Mitogenic activity and EGF radioimmunoassay (RIA) profiles.

of the EGF. Because addition of pepstatin A to the extraction buffer did not alter the yield of EGF, protease inhibitors were not routinely included in the buffers. When the pooled supernatant fluids from the homogenates were made 20% (vol/vol) with respect to acetonitrile and loaded onto a C_{18} silica trace-enrichment column, all of the EGF bound and, when the column was eluted with 3.5 column vol of 50% acetonitrile/H_2O/0.1% CF_3COOH, all of the EGF was recovered. The trace-enriched EGF fraction was diluted with distilled water to reduce the acetonitrile concentration to 20% and loaded directly onto the Ultrasphere ODS column for gradient elution using the C_3F_7COOH/acetonitrile buffer system (Fig. 3).

The protein load was so high that the absorption profile had to be monitored at 305 mm to avoid detector saturation. This reduced the sensitivity of detection by a factor of 1/10 and the ratio of some peaks changed because of their different absorption characteristics. There were two main protein peaks: a broad peak eluting between 36 and 40 min (Fig. 3) that contained the EGF (confirmed by rechromatography of eluate aliquots) and a brown hemoprotein that, from its absorption spectrum and retention time (47.0 min under these conditions), is almost certainly the α-chain of hemoglobin. The high protein loading (15 mg) on this column is responsible for the earlier elution times compared with those of the analytical chromatograms (Fig. 2).

When large loadings of the EGF-enriched fractions from the Ultrasphere ODS column were rechromatographed using a shallow acetonitrile gradient (0.15%/min), the chromatographic efficiency was low. Thus, a large-capacity Waters C_{18} semipreparative column was used for the next stage of the purification (Fig. 4). Partially purified EGF from the Ultrasphere ODS column was resolved into the two major EGF peaks, giving highly purified (>90%) preparations of EGF-α and EGF-β (Fig. 4 *A* and *B*). Purified EGF-α and EGF-β rechromatographed on the C_{18} semipreparative column as distinct peaks having characteristic retention times (26.6 and 28.7 min, respectively). Recombination of the two fractions gave an additive profile (Fig. 4*C*). Likewise, mixtures of purified EGF-α and EGF-β eluted separately under the steeper (1%/min) gradient conditions with the C_3F_7COOH solvent system on the Ultrasphere ODS column but yielded only a single peak when chromatographed at pH 2.1 in 0.1 M sodium phosphate or 0.155 M NaCl.

Final purification was obtained by using a TSK3000SW size exclusion column (Fig. 5). Under these conditions, the size-exclusion mechanism complemented the RP-HPLC methods

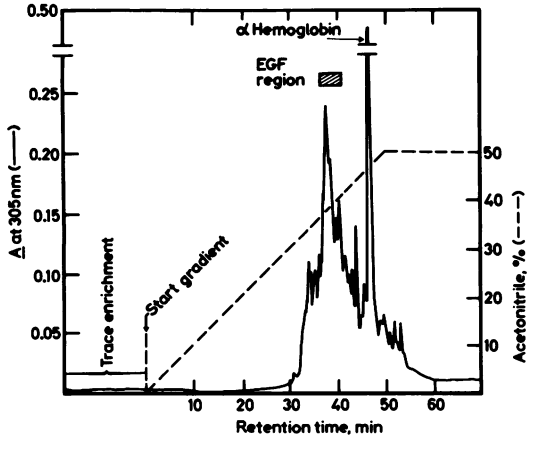

FIG. 3. Preparative RP-HPLC separation of EGF after trace enrichment. The enriched extract (25 ml) from 20 g of salivary glands was made 20% (vol/vol) with respect to acetonitrile and chromatographed under standard conditions on an Ultrasphere ODS column.

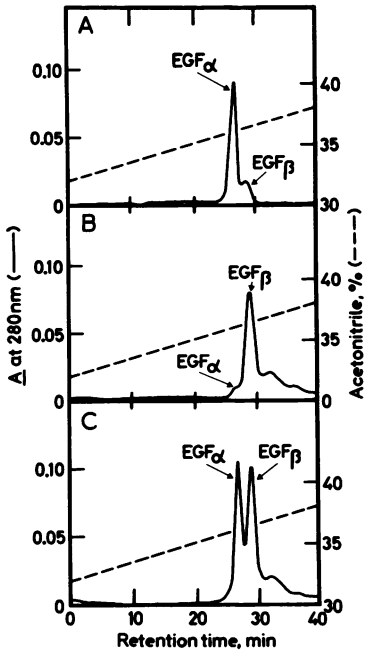

FIG. 4. High-resolution separation of EGF-α and EGF-β by using a semipreparative Waters C_{18} column. (*A* and *B*) Sequential fractions associated with the EGF region from the preparative RP-HPLC (Fig. 3). (*C*) Recombination of fractions of purified EGF-α and EGF-β.

used in the previous stages and allowed resolution of the major EGF peaks (EGF-α and EGF-β, both 12.8 min) from higher molecular weight proteins. Although both EGF-α and EGF-β appeared to be relatively pure when the profiles were monitored at 280 nm (Fig. 5 *A* and *B*), monitoring at 215 nm showed that a contaminant was separating from EGF-β. The EGF eluted later than predicted from its reported molecular weight, inferring increased affinity of EGF for the hydrophilic TSK3000SW silica matrix.

Biological and Chemical Properties of EGF-α and EGF-β. Both the *in vivo* eyelid opening assay and the *in vitro* mitogenic assay indicated that EGF-α and EGF-β were biologically active at similar titers. Newborn mice opened their eyes on day 12 and tooth eruption was evident on day 10. Daily injection of 2 μg

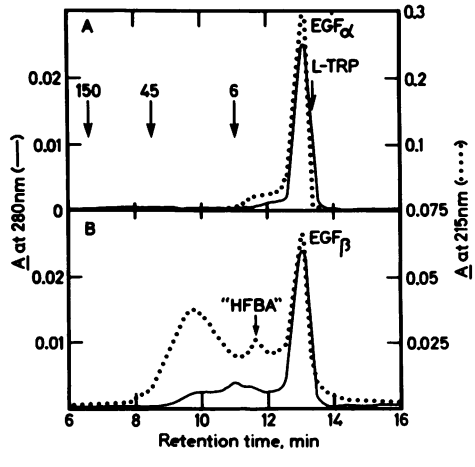

FIG. 5. Size-exclusion chromatography of EGF-α (*A*) and EGF-β (*B*) on TSK3000SW. Proteins were separated at ambient temperature and a flow rate of 1 ml/min on a TSK3000SW column using 0.1 M phosphate buffer (pH 6.5) and detected by UV absorbence at 280 nm (——) or 215 nm (– – –). (Only one-fourth the amount of EGF-β was used to obtain the 215-nm profile.) (The peak labeled "HFBA" is an impurity in the C_3F_7COOH.) ↓, $M_r \times 10^{-3}$.

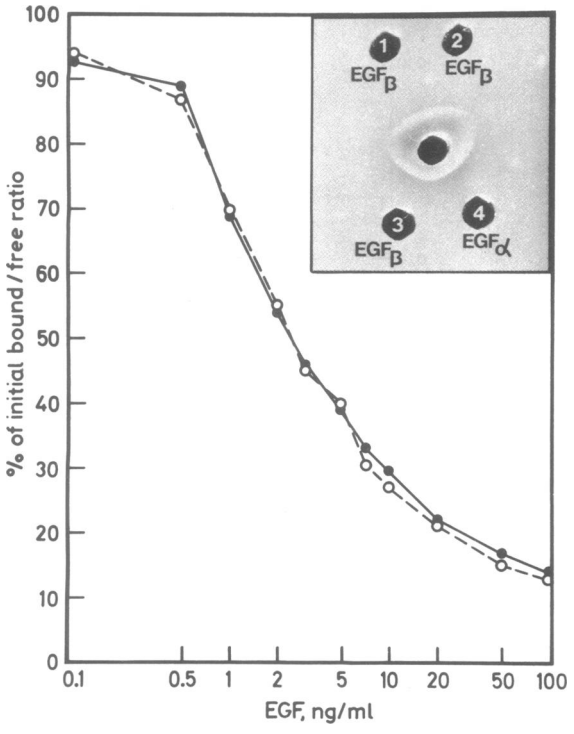

FIG. 6. Competitive radioimmunoassay. ^{125}I-Labeled EGF-α (specific activity, 4×10^8 cpm/μg) competed with EGF-α (●) and EGF-β (○) for binding to wells coated with rabbit anti-EGF serum. Each point represents the mean of quadruplicate determinations. (*Inset*) Immunodiffusion analysis using rabbit anti-EGF serum (center well). Wells: 1, 2, and 3, EGF-β at 0.72, 0.36, and 0.92 mg/ml, respectively; 4, EGF-α at 2.28 mg/ml.

of either EGF-α or EGF-β caused eye opening to occur on day 9 and tooth eruption on day 8. Both EGF-α and EGF-β stimulated the incorporation of [^{3}H]thymidine into 3T3 cell DNA at less than 1 ng/ml. However, maximal stimulation of DNA synthesis with EGF-α occurred at 20 ng/ml whereas EGF-β required more than 50 ng/ml to give maximal stimulation. Immunodiffusion analyses of EGF-α and EGF-β in agarose gels by using rabbit anti-EGF serum (kindly donated by M. Niall) yielded a line of antigenic identity for the two species (Fig. 6). Similarly, both species competed in an identical manner for the binding of ^{125}I-labeled EGF to anti-EGF serum (Fig. 6). The amino acid analyses for EGF-α and EGF-β (Table 1) indicated that the two compositions are almost identical. In addition, NH_2-terminal analyses yielded the same amino acids (aspartic acid, serine) for both EGF-α and EGF-β. The same two end groups were obtained by Cohen and colleagues (18) for the original analysis of the unresolved EGF mixture. These results, together with the different elution characteristics for the proteolytic fragment EGF-2(2) (Fig. 1*C*), indicate that EGF-β is not a proteolytic fragment of EGF-α. Electrophoretic analysis on $NaDodSO_4$/polyacrylamide gels yielded a single band for each protein with an apparent molecular weight of ≈6,000. The ^{125}I-labeled EGFs also electrophoresed as a single species with identical molecular weights. The isoelectric points of EGF-α and EGF-β were identical (pI values of 4.60–4.63), which are in good agreement with the pI reported for EGF (18).

DISCUSSION

Stepwise trace enrichment of EGF on a C_{18} column followed by gradient elution RP-HPLC in the presence of C_3F_7COOH has allowed the rapid isolation of two forms of EGF—EGF-α and EGF-β. After the mouse salivary glands have been extracted, pure EGF-α and EGF-β can be obtained within a few hours. By using a single preparative chromatographic step on a C_{18} ODS column (15 cm × 4.6 mm), more than 10 mg of EGF can be partially separated into EGF-α and EGF-β. The fractions from this column, containing various proportions of EGF-α and EGF-β, can be rechromatographed separately to yield >90% pure EGF-α or EGF-β. A final purification step using a size-exclusion column (TSK3000SW) removes the remaining higher molecular weight impurities from EGF-β. This purification procedure allows direct monitoring of the EGF by its characteristic elution position from RP-HPLC, which is considerably more convenient than radioimmunoassay or mitogenesis procedures. The yield of EGF was 10 mg/20 g (wet weight) of C57BL6/J salivary gland. This represents essentially all of the EGF extracted from the glands and is in agreement with previous estimates for the level of EGF in the salivary glands from this strain of mice (19).

RP-HPLC has been used successfully for the isolation of many peptide hormones (3–5, 20). Our initial use of a low pH sodium phosphate solvent system appeared to allow the separation and purification of EGF. Under these conditions, several preparations of EGF chromatographed as a single sharp symmetrical peak. However, the use of RP-HPLC in conjunction with perfluorinated carboxylic acids such as CF_3COOH and C_3F_7COOH (21) enabled the separation of two distinct species of EGF. The proportion of EGF-α and EGF-β was similar for several different preparations of EGF (both commercial and from different laboratories). In our own laboratories, the proportion of EGF-α and EGF-β did not alter in the presence of protease inhibitors. EGF-β does not seem to be a proteolytic degradation product of EGF-α. Amino acid compositions were very similar. However, the analyses were carried out after acid hydrolysis and there may be differences due to modification.

Table 1. Amino acid compositions of EGF-α and EGF-β

Amino acid	EGF-α	EGF-β	Reported for EGF*	Amino acid	EGF-α	EGF-β	Reported for EGF*
Lys	0	0	0	Ala	0.2	0.6	0
His	1.1	1.1	1	½Cys	6.0	5.0	6
Arg	4.0	3.7	4	Val	1.7	1.9	2
Asp	7.2	5.8	7	Met	0.9	0.9	1
Thr	1.9	1.9	2	Ile	1.7	1.7	2
Ser	6.0	6.1	6	Leu	4.0	3.9	4
Glu	3.0	2.9	3	Tyr	4.9	4.6	5
Pro	2.3	2.1	2	Phe	0	0.1	0
Gly	6.1	6.5	6	Trp†	2.0	2.0	2

Amino acid compositions of EGF-α and EGF-β were estimated from a 24-hr acid hydrolysis.
* Reported in ref. 17 for material purified by using the procedure of ref. 2.
† Determined separately after hydrolysis with methanesulfonic acid (14).

The apparent molecular weights, competitive radioimmunoassay, and immunodiffusion precipitation reactions with anti-EGF serum and the isoelectric points for the two EGFs were identical.

Previous EGF binding studies must have used mixtures of ^{125}I-labeled EGF-α and ^{125}I-labeled EGF-β. Both molecules appear to compete for an identical receptor on the cell surface but we have not yet determined the affinity of binding or the turnover characteristics of the two species.

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