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Murine epidermal growth factor: heterogeneity on high resolution ion-exchange chromatography

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We have shown that epidermal growth factor (EGF) purified either by the classical method of Savage and Cohen, or solely by h.p.l.c. techniques can be resolved into two species, EGFα and EGFβ. However, despite the apparent purity of such materials, as determined both chromatographically and by amino acid analysis, they failed to give homogeneous products on radioiodination. Analysis by isoelectric focusing on agarose gels followed by transfer to nitrocellulose and silver staining showed that EGFα could be further resolved into three sub-species which focused at pH 4.6, 4.3 and 4.1. EGFβ (which also focused at pH 4.6) contained very small amounts of the species with isoelectric points of 4.1 and 4.3, probably due to slight contamination of this preparation by EGFα. Preparative separation of the sub-species of EGFα was achieved by high performance anion-exchange chromatography at pH 6.5 on a Pharmacia Mono Q column. Radioiodination of these purified sub-species did not produce significant charge heterogeneity. However, two slightly different forms of [^{125}I]EGFα_1 (pH 4.6 species) were separable by anion-exchange chromatography on the Mono Q column. All of the EGF species competed for binding to EGF receptors on A431 cells and were active mitogens for BALB/c 3T3 fibroblasts.

Key words: epidermal growth factor/purification/h.p.l.c./heterogeneity

Introduction

Although the epidermal growth factor (EGF), present in the salivary glands of male mice, can be isolated in apparently pure form using gel filtration and ion-exchange chromatography (Savage and Cohen, 1972), there is recent evidence for significant heterogeneity in the final material (Burgess *et al.*, 1982; Matrisian *et al.*, 1982). Using reversed phase high performance liquid chromatography (RP-h.p.l.c.) two forms of EGF (α and β) can be separated. However, these highly purified materials failed to yield homogeneous products on radioiodination. Previous reports have indicated that EGF was modified during radioiodination (Savion *et al.*, 1980; Magun *et al.*, 1982). It is not clear why an iodination procedure which uses Iodogen (Fraker and Speck, 1978; Salacinski *et al.*, 1981) should produce EGF derivatives with such different charges. Whilst Savion *et al.* (1980) and Magun *et al.* (1982) found that the EGF behaved as a single charged species before the radioiodination, our isoelectric focusing analyses indicated that the differently charged EGFs were present before radioiodination. We have therefore attempted to optimise the purification procedure for EGFα and EGFβ by combining the RP-h.p.l.c. with high resolution ion-exchange chromatography (IE-h.p.l.c.) (Soderberg, 1982). Methods are presented for preparing the subspecies EGFα_1, EGFα_2, EGFα_3 and EGFβ which produce essentially homogeneous species when radioiodinated.

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Results and Discussion

Our previous studies (Burgess *et al.*, 1982) suggested that EGF, purified using the method of Savage and Cohen (1972), could be separated into two species EGFα and EGFβ. Initial h.p.l.c. analysis of these materials suggested that they were homogeneous: on both the C18 reversed-phase column (Figure 1A) and the anion-exchange Mono Q column developed at pH 8.0 (Figure 1B) EGFα invariably chromatographed with high efficiency (peak width <30 s at half height). Similar results were obtained with EGFβ. However, numerous attempts to prepare a homogeneous radioiodinated species, chromatographing with similar high efficiency, using Iodogen were unsuccessful. When analysed on the ODS [octadecylsilica] column [^{125}I]EGFα always ran as an ex-

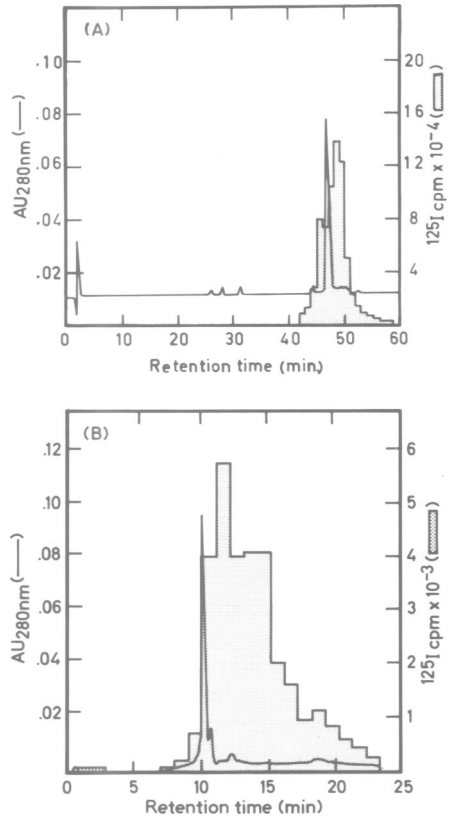

Fig. 1. Reversed phase and anion-exchange chromatograms of partially purified [^{125}I]EGFα. **(A)** RP-h.p.l.c. on Ultrasphere ODS column (15 cm x 4.6 mm i.d.) with linear gradient elution between 0.2% HFBA and 50% CH_3CN/0.2% HFBA over 50 min. **(B)** IE-h.p.l.c. on Pharmacia Mono Q column with linear gradient elution between 20 mM Tris-HCl pH 8.0 and 20 mM Tris-HCl pH 8.0 containing 0.3 M NaCl over 15 min. Both chromatograms were developed at ambient temperature and a constant flow of 1 ml/min. The 280 nm absorbance profile (———) of purified EGFα carrier is compared with the radioactivity profile (▨) of 1 ml eluant fractions from the corresponding radioiodinated material.

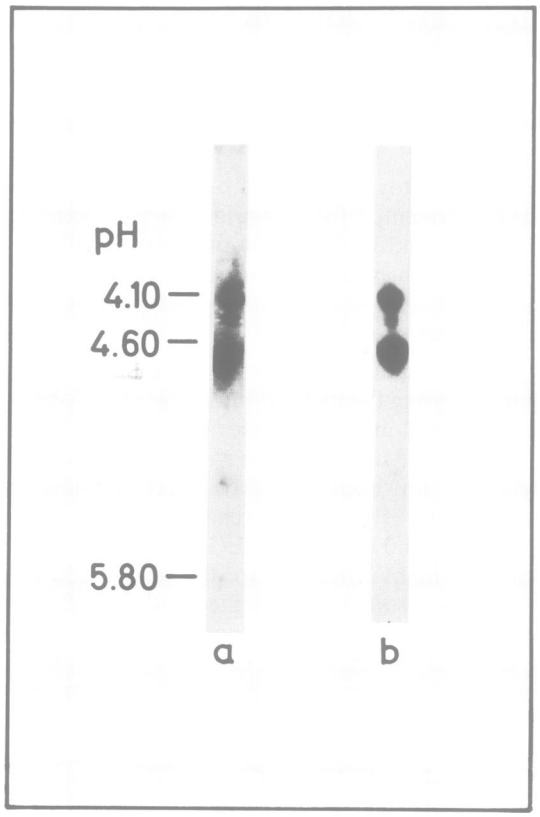

Fig. 2. Isoelectric focusing analysis of EGFα and [^{125}I]EGFα. After transfer to nitrocellulose the filter was washed and the EGF proteins detected using a modified silver stain (Nicola, in preparation) – **lane a**; and autoradiographed to detect the [^{125}I]EGF species – **lane b**.

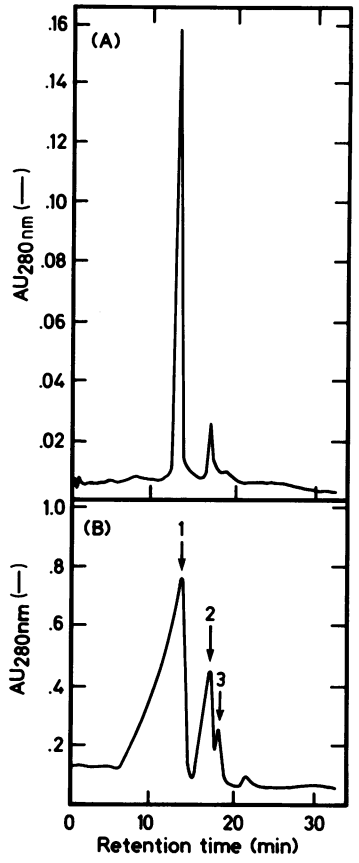

Fig. 3. Analytical (**A**) and preparative (**B**) separation of EGFα$_1$, EGFα$_2$ and EGFα$_3$. (**A**) 100 μg of EGFα chromatographed on a Pharmacia Mono Q column. The column was equlibrated with bis-Tris-HCl (20 mM, pH 6.5) and proteins eluted with a NaCl gradient of 2.5 mM/min at a constant flow of 1 ml/min. (**B**) 1.5 mg of EGFα chromatographed under the same conditions. Aliquots of the peaks indicated were further analysed by isoelectric focusing (see Figure 4).

tremely broad peak (width at half height >5 min, Figure 1A). However, the relative binding affinity of [^{125}I]EGFα to A431 carcinoma cells was similar in fractions recovered across this peak of radioactivity (data not shown). Similar results were found when the Mono Q ion-exchange column was used to analyse [^{125}I]EGFα (Figure 1B) or [^{125}I]EGF purchased from Amersham, which is prepared from EGF purified by the classical method of Savage and Cohen (1972). [^{125}I]EGF derivatives always eluted in a broad region compared with unlabeled EGF although recovery of radioactivity was quantitative. The fact that the unlabeled protein could always be reproducibly chromatographed with high efficiency and recovery suggested that the poor efficiency of the chromatographic profiles of the radiolabeled EGFs was due to multiple molecular species rather than non-specific adsorption of the protein onto the chromatographic matrices or conformational changes due to the chromatographic solvents. Although some oxidation of methionine or tryptophan could occur during iodination (Salacinski *et al.*, 1981) the generation of derivatives with an apparently wide charge distribution (Figure 1B) is difficult to understand.

Our analyses of the EGFs using isoelectric focusing were made with agarose gels. Attempts to fix the EGF (using acid-methanol) for subsequent staining with Coomassie Blue R250 were unsuccessful, as both EGFα and EGFβ appeared to be soluble in the fixative. However, by focusing larger amounts of EGFα or EGFβ a precipitated band was evident at pH 4.6. If the focused gel was fixed with aqueous trichloroacetic acid/sulphosalicylic acid (10% v/v and 5% w/v, respectively) it was possible to detect a major EGF band at pH 4.6 which stained weakly with Coomassie Blue R250.

These results were in agreement with Magun *et al.* (1982) and Savion *et al.*. (1980) who reported that their unlabeled EGF preparations focused as a single band in polyacrylamide gels. However, both of these reports also indicated that under the same conditions [^{125}I]EGF focused as several species. They suggested that these species were generated by the iodination procedure.

For quantitative analysis of EGF after isoelectric focusing in agarose gels, it was necessary to avoid the loss of any species during the fixation procedure. Transferring the proteins to nitrocellulose after focusing between pH 3.5 and pH 5.9 was ideal for analysing the [^{125}I]EGF species by subsequent autoradiography. To optimize such manipulations, [^{125}I]EGF purchased from Amersham was focused, transferred and autoradiographed in this way. Four radioiodinated bands were apparent between pH 4.10 an pH 4.6. The transfer was essentially quantitative with <1% of the [^{125}I]-EGF remaining associated with the agarose and 5% diffusing through the nitrocellulose to the filter paper backing. The [^{125}I]EGFs detected were very similar to the species reported by Magun *et al.* (1982). However, initial attempts to transfer unlabeled EGF after isoelectric focusing were further complicated by the precipitation of EGFα at pH 4.6. Although some transfer from agarose to nitrocellulose took place, most of the precipitated EGFα remained associated with the

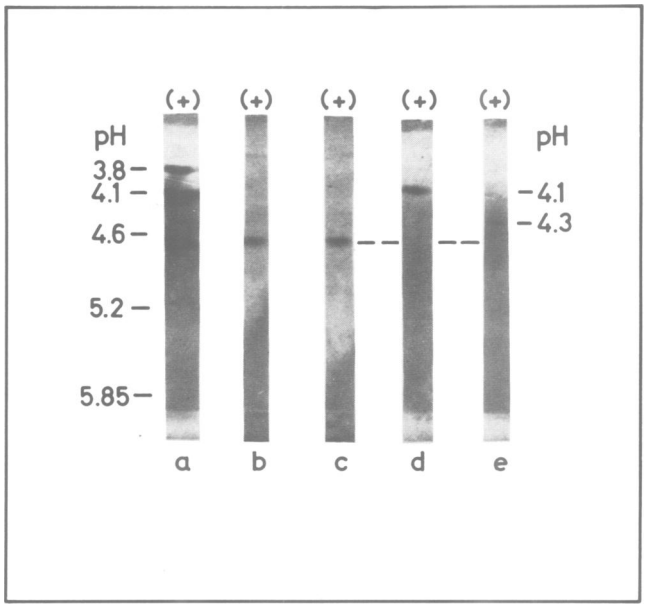

Fig. 4. Isoelectric focusing analyses of EGFα and EGFβ sub-species prepared by ion-exchange chromatography (Figure 3B). EGF protein was transferred to nitrocellulose and detected by the silver stain method. **Lane a**, unfractionated EGFα (500 ng); **lane b**, EGFβ (200 ng) prepared by ion-exchange chromatography on a mono Q column at pH 6.5; **lanes c, d, e**, peaks 1 (200 ng), 2 (200 ng) and 3 (100 ng) respectively from EGFα chromatographed on Mono Q column at pH 6.5 (Figure 3B).

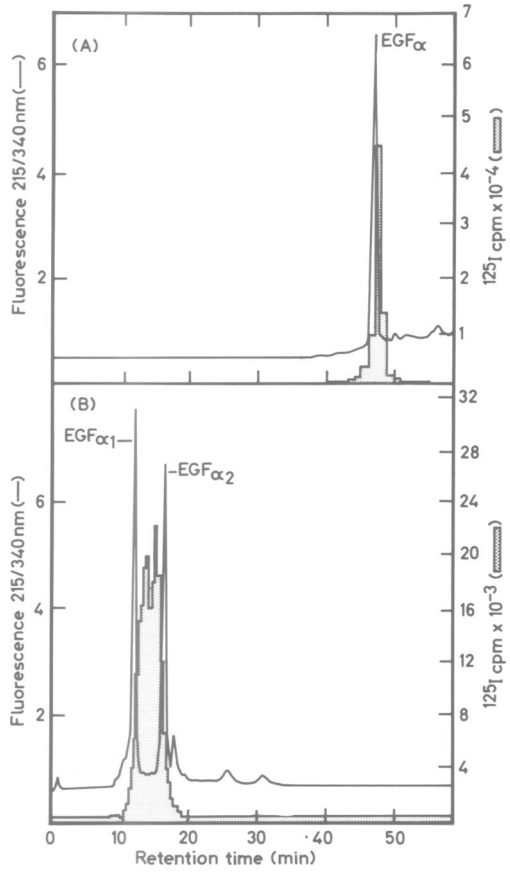

Fig. 5. Reversed phase and anion-exchange chromatograms of highly purified [^{125}I]EGFα_1 (pI 4.6). **(A)** RP-h.p.l.c. on Ultrasphere ODS (15 cm x 4.6 mm i.d.) with gradient elution conditions as for Figure 1A. **(B)** IE-h.p.l.c. on Pharmacia Mono Q. Chromatographic conditions were as for Figure 3. The [^{125}I]EGFα_1 was mixed with 1 μg of unlabeled EGFα_1 and 1 μg of EGFα_2 for this chromatogram. The radioactivity profiles (▨) of eluant fractions are compared with the fluorescence profile (215 nm excitation/ 340 nm emission) (———) of EGFα carrier.

agarose gel. When urea (2 M) was added to the agarose gel no precipitate was formed and the transfer of both unlabeled EGF and [^{125}I]EGF to the nitrocellulose was >90%. When the urea concentration was increased to 4 M, >30% of the [^{125}I]EGF passed through the nitrocellulose to the filter paper beyond. All of our subsequent isoelectric analyses of EGF were performed on agarose gel containing urea (2 M) and the gels were prefocused for 30 min before loading the sample. The transfer of unlabeled EGF to nitrocellulose filters allowed the use of a sensitive detection method, namely the silver stain (Morrissey *et al.*, 1981). This method, as modified for nitrocellulose by Nicola (in preparation), was able to detect rapidly 50 ng of protein. When h.p.l.c. purified EGFα (Burgess *et al.*, 1982) and the corresponding [^{125}I]EGFα were analysed using this method, three identical protein bands were seen on both the silver-stained filter and the autoradiograph of the [^{125}I]EGFα (Figure 2). The abundance of the three protein bands (at pH 4.6, 4.3 and 4.1) was apparently identical for both the EGFα and [^{125}I]EGFα, suggesting that the iodinated products resulted from the labeling of proteins present at the time of radioiodination, rather than by the production of new derivatives during the iodination procedure. This notion was supported by the constant pattern of labeling under different conditions (e.g., increased Iodogen, longer times of labeling, different I^- concentrations). Although the amount of ^{125}I incorporated into EGFα was varied 50-fold, the proportion of the differently charged species did not alter significantly.

In an attempt to separate these different species of EGFα the Mono Q column was developed with shallow salt gradient buffered with 20 mM bis-Tris-HCl at pH 6.5. The EGFα species chromatographed with high efficiency, and under these conditions the major species were separated by >4 min (Figure 3A). This procedure was suitable for preparing milligram quantities of the individual EGF sub-species (Figure 3B), although in the preparative mode considerable chromatographic band broadening was evident. The unfractionated EGFα, and the separated EGF peaks 1, 2 and 3 from the Mono Q column (Figure 3B) were analysed by isoelectric focusing, blotted onto nitrocellulose and the protein detected with the silver stain (Figure 4). The resolved peaks from the Mono Q column contained the different sub-species of EGFα: EGFα_1 (pI 4.6), EGFα_2 (pI 4.1), EGFα_3 (pI 4.3). Using analytical IE-h.p.l.c. with the Mono Q column developed at pH 6.5 the proportion of EGFα_1 and EGFα_2 could be shown to vary across the preparative reversed phase column profile obtained in our earlier studies (Figure 3, Burgess *et al.*, 1982): at the top of the EGF peak only a small proportion of EGFα_2 was found whereas at the trailing edge of this peak the EGFα_1:EGFα_2 ratio approached 1:1. All of the commercial EGFs and EGF prepared by the method of Savage and Cohen (1972) contained both EGFα_1 and EGFα_2. Preliminary evidence indicated that above neutrality, in mixtures containing both EGFα_1 and EGFα_2, there was a gradual conversion of EGFα_1 to EGFα_2. However in highly purified EGFα_1 preparations stored at 4°C at pH 6.5 we have not observed conversion of EGFα_1 to EGFα_2 or EGFα_3. Although EGFβ is almost certainly present in the murine salivary gland (Burgess *et al.*, 1982) it is possible that EGFα_2 and EGFα_3 are degradation products of EGFα_1. We are presently investigating dif-

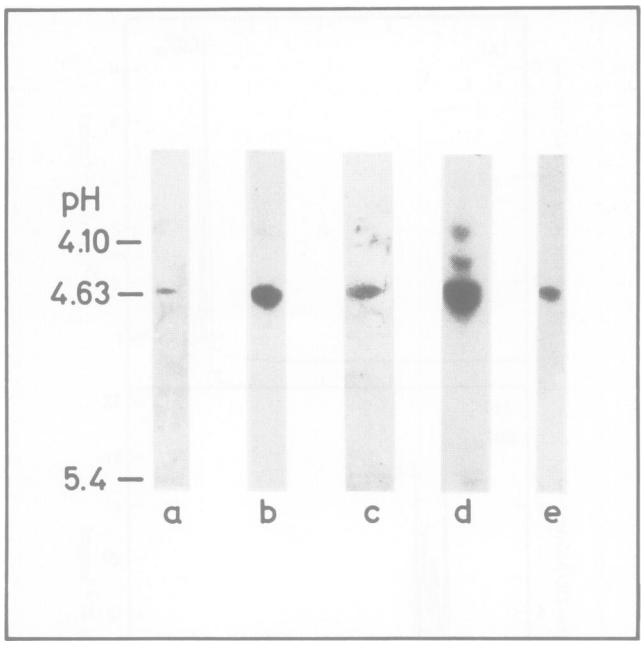

Fig. 6. Isoelectric focusing analyses of IE-h.p.l.c. purified EGF sub-species and their corresponding radioiodinated derivatives. **Lane a**, EGFα_1 (100 ng), protein silver stain; **lane b**, [^{125}I]EGFα_1, autoradiograph; **lane c**, EGFβ (200 ng), protein silver stain; **lane d** [^{125}I]EGFβ (iodinated before purification using the Mono Q column at pH 6.5); and **lane e**, [^{125}I]EGFβ (iodinated after purificaton using the Mono Q column at pH 6.5), autoradiograph.

ferent purification protocols to establish whether EGFα_2 or EGFα_3 are formed during the acid extraction or chromatography.

EGFβ also contained material which focused at pH 4.1 and 4.3, but the proportion of these species was always $< 5\%$ of the total protein and may well have represented minor contamination of the EGFβ with EGFα due to incomplete resolution of these species in our earlier studies (Burgess *et al.*, 1982).

EGFα_1 purified by h.p.l.c. on the Mono Q column at pH 6.5 could be iodinated using $^{125}I^-$ and Iodogen to yield a labeled derivative (~ 250 μCi/μg) which chromatographed with high efficiency on both a reversed phase C_{18} column (Figure 5A) and a Mono Q ion-exchange column (Figure 5B). On the reversed phase column, the majority of the radiolabel was recovered in a single 1 min fraction eluting 1–2 min after EGFα_1 carrier (Figure 5A). Similar results were obtained with EGFα_2, EGFα_3 and EGFβ. This is in marked contrast to the broad radioactivity profile obtained under identical chromatographic conditions with [^{125}I]EGFα prepared from material not subjected to the ion-exchange fractionation (Figure 1A). On the ion-exchange column (Figure 5B) [^{125}I]-EGFα_1 eluted between EGFα_1 and EGFα_2 and there was a suggestion of two peaks of ^{125}I, although these were not completely resolved. Analysis of [^{125}I]EGFα_1, [^{125}I]EGFβ (iodinated before IE-h.p.l.c.) and [^{125}I]EGFβ [iodinated after purification on IE-h.p.l.c. (cf Figure 3A)] by isoelectric focusing is shown in Figure 6. The highly purified EGFα_1, EGFβ, [^{125}I]EGFα_1 and [^{125}I]EGFβ focused as single species. The EGFβ which had not been purified on the Mono Q column at pH 6.5 contained small amounts of material focusing at pH 4.1 and 4.3 ($< 5\%$ of the total protein) which were detected after iodination. The [^{125}I]EGFα_1 and [^{125}I]EGFβ autoradiographic bands were often slightly broader than the silver-stained bands for the unlabeled EGFα_1 and EGFβ, respectively. This could be due to the production of different ^{125}I forms, e.g., mono- and di-iodo-derivatives. Such slightly different iodination derivatives could explain the two overlapping peaks of [^{125}I]EGFα_1 observed on the Mono Q column (Figure 5B).

Table I. Receptor binding and mitogenic activity of EGF sub-species

EGF sub-species	Binding affinity[a]	Mitogenic activity[b]
Unfractionated EGFα	ND	0.58
EGFα_1	76	0.29
EGFα_2	62	0.33
EGFα_3	55	1.00
EGFβ	84	0.38

[a]The EGF concentration (ng/ml) causing 50% [^{125}I]EGFα_1 displacement from A431 cells.
[b]The EGF concentration (ng/ml) causing half maximal stimulation of DNA synthesis in BALB/c 3T3 cells.

All of the sub-species are active mitogens for murine 3T3 cells and all bind to EGF receptors on A431 carcinoma cells (Table I). The binding curves for EGFα_1, EGFα_2, EGFα_3 and EGFβ are similar. After quantitating the concentration of each EGF sub-species using either optical density at 280 nm (Taylor *et al.*, 1972) or quantitative analysis of fluorescence using the h.p.l.c. detector, all sub-species could be shown to displace 50% of the [^{125}I]EGFα_1 at concentrations between 55 and 84 ng/ml (Table I). Similarly, the concentration of EGFα_1, EGFα_2 and EGFβ required to stimulate half maximal incorporation of [^{3}H]thymidine into DNA was between 290 and 380 pg/ml. EGFα_3 had lower mitogenic activity (1000 pg/ml). Unfractionated EGFα caused half maximal stimulation at 580 pg/ml. This latter result is in contrast to the findings of Matrisian *et al.* (1982) who report a greatly increased (10-fold) mitogenic activity of their purified EGFα sub-species.

Using the techniques reported herein, it is now possible to prepare rapidly sufficient quantities of EGFα_1 and EGFβ for both crystallographic and spectroscopic studies. It is hoped that the availability of these highly purified species will allow the three-dimensional structure of EGF to be studied in more detail. The preparation of homogeneous preparations of [^{125}I]EGF derivatives should facilitate studies of the processing of EGF by its target cells where multiple charge separable species are known to be formed (Magun *et al.*, 1982).

Materials and methods

H.p.l.c. preparation of EGFα and EGFβ

The initial purification of EGFα and EGFβ using RP-h.p.l.c. was performed as described previously (Burgess *et al.*, 1982) with the following modification: the final h.p.l.c. gel filtration stage on TSK3000SW was replaced by high resolution anion-exchange chromatography on a Mono Q column (Pharmacia, Sweden). The EGFα or EGFβ fractions from the reversed phase column were diluted 10-fold with bis-Tris-HCl (20 mM, pH 6.5) and loaded onto the Mono Q column equilibrated with the same buffer. The column was then further equilibrated with 10 ml of buffer before eluting the proteins with a linear NaCl gradient of 2.5 mM/min at a constant flow rate of 1 ml/min. Proteins were detected by both their u.v. absorption at 280 nm and endogenous tryptophan fluorescence (Schoeffel FS950 detector 215 nm excitation, 340 nm emission).

Iodination of EGF sub-species

5 μg of the EGF (50 μl) to be radioiodinated was mixed with one-fifth

volume of Tris-HCl (100 mM, pH 7.4) and 1 mCi Na ^{125}I (10 μl, carrier-free, from Amersham, UK) and pipetted into a polypropylene tube coated with 10 μg of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, Pierce Chem. Co., IL). After 10 min the mixture was diluted into 500 μl of 20 mM sodium phosphate buffer, pH 7.4 containing NaCl (0.155 M) (Salacinski *et al.*, 1981). The solution was allowed to stand for 15 min before separating the [^{125}I]EGF and free $^{125}I^-$ using either Sephadex G-25 (Pharmacia PD-10 column) eluted with phosphate buffered saline, or a Waters C_{18} Sep-pak cartridge equilbrated with heptafluorobutyric acid (HFBA), and eluted with acetonitrile-water (50% v/v). The [^{125}I]EGF sub-species prepared by this method had a specific activity of ~250 μCi/μg.

Isoelectric focusing

Isoelectric focusing was performed on thin layer agarose gels (Grubb, 1973; Olsson and Laas, 1981) containing urea (2 M). The pH gradient was formed using equal parts of Pharmalyte 2.5–5 and Pharmalyte 4–6.5 (Pharmacia, Sweden). The agarose (Pharmacia IEF grade) solution (1% w/v) in distilled water containing sorbitol (12 g/100 ml) was boiled before adding urea (12 g/100 ml) and the Pharmalytes (3.25 ml of each/100 ml). This mixture was poured onto the hydrophilic surface of a Gelbond plastic sheet (110 x 205 mm, Marine Colloid Co., ME) and after setting, stored in a fully humidified box. The gels were prefocused for 30 min at 3 W constant power (to remove ionic species which could modify the samples) before loading the samples (20 μl) via Whatman No. 1 filter paper and focusing for a further 30 min at 10 W. The sample applicators were removed and the focusing continued at 15 W for 30 min. The focused proteins were transferred to nitrocellulose (Burnette, 1981; Reinhart and Malamud, 1982), stained with silver to detect the proteins (Morrissey *et al.*, 1981; Nicola, in preparation) and autoradiographed to detect the ^{125}I-labeled derivatives. Autoradiographs or silver-stained protein bands were quantitated using an optical data digitiser (Malachowski *et al.*, in preparation).

Receptor binding assay

Receptor binding affinity was determined using A431 carcinoma cells (Haigler *et al.*, 1978) which were kindly provided by G. Gill (Salk Institute, CA). Cells were grown to confluence in 35 mm culture dishes (~1.5 x 10^6 cells). The assay was performed in 2 ml of Dulbecco's modified Eagles medium (DME) containing 0.1% bovine serum albumin (BSA). Cells were incubated for 90 min at 37°C in the presence of [^{125}I]EGFα_1 (100 000 c.p.m., 445 000 c.p.m./ng) and the competing unlabeled EGF sub-species (1–500 ng/ml). At the end of the labeling period the cells were washed 5 times with ice cold phosphate buffered saline (0.02 M sodium phosphate, 0.15 M NaCl) containing BSA (0.1% w/v) before solubilisation in 2 ml NaOH (0.5 M). Radioactivity was determined using a Packard Model 500C γ spectrometer. The relative binding affinities of different [^{125}I]EGF fractions across the RP-h.p.l.c. profile for EGFα (iodinated before IE-h.p.l.c.) (Figure 1A) were determined using the same assay.

Mitogenic assay

BALB/c 3T3 fibroblasts were grown to confluence in 16 mm cluster plates in DME containing 10% fetal calf serum. Prior to assay the cells were washed and then incubated with DME containing 0.5% fetal calf serum (1 ml) and the respective EGF sub-species (0.1–5 ng/ml) for 20 h at 37°C. The cells were pulsed with [^{3}H]thymidine (1 μCi/ml, 100 Ci/mol) for 2 h before washing 3 times with phosphate buffered saline, solubilisation in 0.5 M NaOH (0.5 ml) and precipitation with ice cold 20% trichloroacetic acid (TCA, 0.5 ml). The precipitate was recovered by filtration onto glass fibre discs, washed twice with 10% TCA, the discs dried with ethanol and the recovered radioactivity determined by liquid scintillation counting.

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