Methods in Enzymology

Volume 146

Peptide Growth Factors

Part A

EDITED BY

David Barnes

DEPARTMENT OF BIOCHEMISTRY
AND BIOPHYSICS
OREGON STATE UNIVERSITY
CORVALLIS, OREGON

David A. Sirbasku

DEPARTMENT OF BIOCHEMISTRY
AND MOLECULAR BIOLOGY
UNIVERSITY OF TEXAS MEDICAL SCHOOL
HOUSTON, TEXAS

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
Orlando San Diego New York Austin
Boston London Sydney Tokyo Toronto

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS INC. (LONDON) LTD.
24–28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 54-9110

ISBN 0–12–182046–7 (alk. paper)

PRINTED IN THE UNITED STATES OF AMERICA

87 88 89 90 9 8 7 6 5 4 3 2 1

Table of Contents

Section I. Epidermal Growth Factor

Section II. Transforming Growth Factors

Section III. Somatomedin/Insulin-Like Growth Factors

Section IV. Bone and Cartilage Growth Factors

Section V. Techniques for the Study of Growth Factor Activity: Assays, Phosphorylation, and Surface Membrane Effects

Section I

Epidermal Growth Factor

[1] Purification of Human Epidermal Growth Factor by Monoclonal Antibody Affinity Chromatography

By ROBERT A. HARPER, JAMES PIERCE, and C. RICHARD SAVAGE, JR.

Introduction

Epidermal growth factor (EGF) is a single chain polypeptide containing 53 amino acid residues that exhibits potent mitogenic activity for a variety of cell types both *in vivo* and *in vitro*. Many reviews are available concerning the mechanism of action and biological effects of EGF.[1-5] This molecule was first described by Cohen,[6] who isolated EGF from the submaxillary glands of adult male mice (mEGF). Since then EGF has been isolated from the rat,[7] the guinea pig prostate,[8] and human urine (hEGF).[9-11]

At the time Cohen[9] first reported the amino acid composition of hEGF in 1975, Gregory[12] simultaneously reported the structure of the antisecretory agent "urogastrone" which is also found in urine. Gregory demonstrated that urogastrone and EGF has similar amino acid structures as well as biological properties. From this early work of Cohen and Gregory it was soon clear that these two molecules were one and the same. A comparison of the primary structure of mouse EGF[13] and human EGF[12] is shown in Fig. 1. There exist a remarkable degree of sequence homology between hEGF and mEGF with 37 of the 53 amino acid residues comprising the two molecules being identical and with the three disulfide linkages located in the same relative positions. Only 2 of the remaining 16 nonhomologous

[1] G. Carpenter, *in* "Handbook of Experimental Pharmacology" (R. Baserga, ed.), p. 89. Springer-Verlag, New York, 1981.
[2] G. Carpenter and S. Cohen, *Annu. Rev. Biochem.* **48**, 193 (1979).
[3] M. Das, *Int. Rev. Cytol.* **78**, 233 (1982).
[4] D. Gospodarowicz, *Annu. Rev. Physiol.* **43**, 251 (1981).
[5] H. Haigler, *in* "Growth and Maturation Factors" (G. Guroff, ed.), p. 117. Wiley, New York, 1983.
[6] S. Cohen, *J. Biol. Chem.* **237**, 1555 (1962).
[7] J. B. Moore, Jr., *Arch. Biochem. Biophys.* **189**, 1 (1978).
[8] J. A. Rubin and R. A. Bradshaw, *in* "Methods for Preparation of Media, Supplements and Substrata for Serum-Free Animal Cell Culture" (D. W. Barnes, O. A. Sirbasku, and G. H. Sato, eds.), p. 139. Liss, New York, 1984.
[9] S. Cohen and G. Carpenter, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1317 (1975).
[10] H. Gregory and I. R. Willshire, *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 1765 (1975).
[11] C. R. Savage, Jr., and R. A. Harper, *Anal. Biochem.* **111**, 195 (1981).
[12] H. Gregory, *Nature (London)* **257**, 325 (1975).
[13] C. R. Savage, Jr., T. Inagami, and S. Cohen, *J. Biol. Chem.* **247**, 7612 (1972).

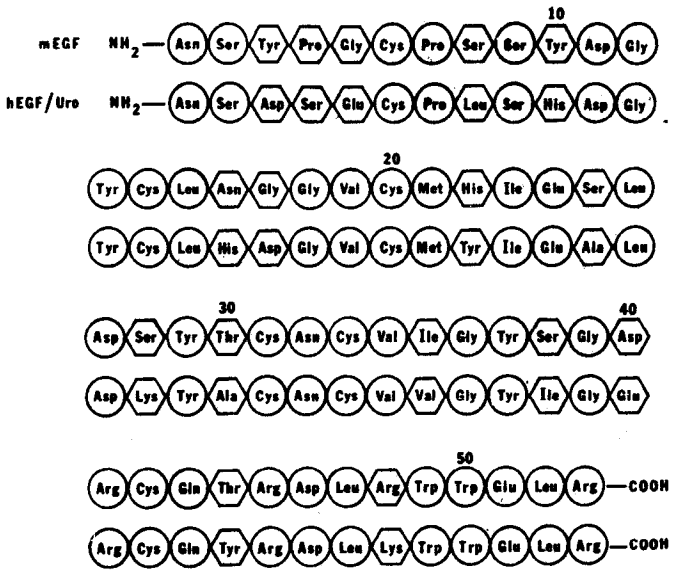

FIG. 1. Primary structures of mouse and human EGF. Amino acid residues shown in circles are identical; amino acid residues shown in hexagons are nonidentical.

amino acid residues cannot be interconverted by a single base change in the triplet code. It is also estimated that guinea pig EGF has a 70% homology with the sequences of mouse and human EGF.[8]

The most abundant source of EGF known is the submaxillary glands of the mouse. It is present in the gland and at levels up to 0.5% of the dry weight. Milligram quantities of EGF can be purified readily from the mouse[14]; however, this is not the case for hEGF. In 1975 Cohen and Carpenter[9] published a procedure for the purification of hEGF using concentrates of urinary proteins (not raw urine). That same year Gregory and Willshire[10] reported a procedure which involved the fractionation of up to 3000 liters of human urine utilizing a 12-step procedure which resulted in relatively low yields of hEGF (3–5%). In 1981[11] our laboratory reported a purification procedure for hEGF using 20 liters of raw urine which was less time consuming and resulted in good yields of highly purified hEGF. We have subsequently refined this method to incorporate the use of a rabbit polyclonal antibody affinity column.[15]

[14] C. R. Savage, Jr., and S. Cohen, *J. Biol. Chem.* **247**, 7609 (1972).

[15] C. R. Savage, Jr., and R. A. Harper, *in* "Methods for Preparation of Media, Supplements and Substrata for Serum-Free Animal Cell Culture" (D. W. Barnes, O. A. Sirbasku, and G. H. Sato, eds.), p. 147. Liss, New York, 1984.

In this chapter we would like to summarize part of our standard published procedure and present a modification that employs the use of monoclonal antibody affinity chromatography. We will discuss the use of monoclonal antibody affinity chromatography in the light of other published reports using this technology. (See also Nishikawa *et al.*, this volume [2].)

Materials and Methods

Human Epidermal Growth Factor or Urogastrone

Five liters of adult human urine is collected from the first morning void, acidified with 250 ml of glacial acetic acid, and the pH adjusted to 3.0 with concentrated HCl. This represents the starting material for the purification procedure described in this chapter.

Monoclonal Antibody to hEGF, 836.D4

The monoclonal antibody (836.D4) used for the purification of hEGF from urine is obtained by fusing murine myeloma cells with BALB/c mouse splenocytes sensitized to hEGF. The full characterization of the monoclonal antibody has been published previously.[16] The antibody does not react with either rat or mouse EGF or with 11 other polypeptide hormones tested as shown by solid phase radioimmunoassay and immunoprecipitation followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Scatchard analysis of the antibody binding to purified hEGF reveals an apparent equilibrium dissociation constant of 1×10^{-8} *M*. The antibody blocks both the binding of hEGF and hEGF stimulation of [^{3}H]thymidine incorporation into DNA by >90% in confluent cultures of human foreskin fibroblasts.

Purification of Monoclonal Antibody by DEAE Affi-Gel Blue Chromatography

Ascites fluid is centrifuged at 2000 *g* for 5 min to remove cells and then centrifuged at 100,000 *g* for 30 min. The fluid is then dialyzed overnight at 4° against 100 vol of 0.02 *M* Tris–HCl, pH 7.2. Purification of the monoclonal antibody is carried out according to Bruck *et al.*[17] All procedures are carried out at 4°. One milliliter of ascites fluid is applied to a 7-ml column (a 10-ml disposable CoStar plastic pipet) of DEAE-Affi-Gel Blue

[16] D. M. Moriarity, R. A. Harper, B. B. Knowles, and C. R. Savage, Jr., *Hybridoma* 2, 321 (1983).

[17] C. Bruck, D. Portetelle, C. Glineur, and A. Bollen, *J. Immunol. Methods* 53, 313 (1982).

(Bio-Rad, Richmond, CA). The column is washed with 3 column volumes of 0.02 *M* Tris–HCl, pH 7.2, and then with 3 column volumes of the column buffer containing 25 m*M* NaCl. The monoclonal antibody is then eluted with 3 column volumes of buffer containing 50 m*M* NaCl. The flow rate is approximately 30 ml/hr and 4-ml fractions are collected. The eluted fractions are tested for monoclonal antibodies directed toward hEGF by our previously published solid phase radioimmunoassay.[16]

Radioreceptor Assay for hEGF

The assay is conducted in 24-multiwell tissue culture plates using confluent monolayers of human skin fibroblasts as previously described.[11] Briefly, a standard curve is generated in a final volume of 0.2 ml of binding medium containing 0.5 mg of ^{125}I-labeled mEGF ($\sim 1 \times 10^5$ cpm) and 0 to 10 ng of nonlabeled mEGF. After 40 min at 37° the binding medium is removed and the cells washed four times with ice-cold buffer. The plates are drained, the cells are dissolved in 1.0 ml of 10% NaOH for 10 min, and the amount of radioactivity bound is then determined. Nonspecific binding is determined in the presence of 1000-fold excess of nonlabeled mEGF. This value, which is less than 6% of the total amount bound, is subtracted from each point. Using this assay, we can routinely measure 0.5 ng of hEGF.

Preparation of the Monoclonal Antibody Affinity Gel

Monoclonal antibody 836.D4 is coupled to Affi-Gel 10 (Bio-Rad; Richmond, CA) according to the manufacturer's protocol. In short, 2 ml of gel is washed three times with ice-cold isopropanol and then three times with ice-cold distilled water. Purified monoclonal antibody (20 mg) is added to the gel in 0.12 *M* HEPES, pH 7.5, containing 0.15 *M* NaCl and the mixture is rotated overnight at 4°. The Affi-Gel 10 is sedimented by centrifugation and treated at room temperature for 1 hr with 1 *M* ethanolamine, pH 8.0, in order to block any active esters which might remain. The Affi-Gel is then washed extensively with 0.05 *M* phosphate buffer, pH 8.0, containing 0.15 *M* NaCl, and stored at 4° in the same buffer containing 0.1% NaN_3 as preservative.

Results

Premonoclonal Antibody Affinity Column Purification Steps

Step 1: Batch Bio-Rex 70. Five liters of adult human urine contain approximately 150–200 μg of hEGF as determined by the radioreceptor

TABLE I
PURIFICATION OF HUMAN EGF FROM URINE

Procedure	Total volume (ml)	Concentration of hEGF (μg/ml)[a]	Total hEGF (μg)	Average yield (%)
Urine	5000	0.03–0.04	150–200	100
Batch Bio-Rex 70	12[b]	12–18	144–216	100
Ethanol precipitation	10	15–20	150–200	100
Passage over DE-52 Cellulose	10[b]	14–18	140–180	>90
Monoclonal antibody affinity column	1	80–103	80–103	52

[a] Determined by the radioreceptor assay, assuming that hEGF and mEGF compete equally for binding of ^{125}I-labeled mEGF to the membrane receptor on human skin fibroblasts.

[b] Indicates volumes after lyophilization and resuspension of the dry residue. This was necessary since the presence of high concentrations of ammonium acetate interfere with the radioreceptor assay.

assay (Table I). At the outset, we attempted to adsorb the EGF directly from the urine using the monoclonal antibody coupled to Affi-Gel 10. We were unsuccessful in getting the EGF to bind to the affinity matrix. We therefore decided to use the first three steps in our previously published procedure in order to remove large amounts of brown–black pigments and protein.

Briefly, Bio-Rex 70 ion-exchange resin (Bio-Rad; Richmond, CA) is suspended in water and the pH is adjusted to 3.0 with glacial acetic acid. Sixty milliliters of settled resin is added to the 5 liters of urine and the mixture is stirred for 18 hr at 4°. The resin is then allowed to settle and the supernatant fraction discarded. The resin is transferred into a 4-cm-diameter glass column and washed with 3.6 liters of 10^{-3} *N* HCl at 25°. The EGF is then eluted with 240 ml of 1.0 *M* ammonium acetate, pH 8.0. The eluate, containing 144–216 μg of EGF, is then lyophilized (Table I).

Step 2: Ethanol Precipitation. Pepstatin (0.5 mg) is added and the dry residue is suspended at 25° in 10 ml of a 2 m*M* aqueous solution containing 50 mg of crystalline bovine serum albumin. To this mixture 125 ml of absolute ethanol is added. The resulting precipitate is collected by centrifugation and the clear brown supernatant fraction is discarded. The pellet is suspended in 5 ml of 2 m*M* arginine and the pH is adjusted to 3.0. The mixture is recentrifuged and the clear dark brown supernatant fraction (10 ml) containing 150–200 μg of EGF (Table I) is kept.

Step 3: Passage over DE-52 Cellulose. A 4 × 5 cm column of DE-52 cellulose (Whatman) is prepared and equilibrated at 25° with 0.05% formic acid, pH 3.0. The supernatant fraction from the previous step is applied to the column and the column washed with 135 ml of 0.05% formic acid, pH 3.0. Under these conditions the EGF is not adsorbed to the cellulose. The column effluent is collected on ice and then lyophilized. The residue is suspended in 10 ml of 0.05 *M* phosphate-buffered saline, pH 8.0. The mixture is centrifuged and the brown supernatant fraction containing 140–180 μg EGF is retained. Through this stage of purification greater than 90% of the starting amount of EGF is recovered (Table I).

Monoclonal Antibody Affinity Column. All procedures are carried out at room temperature. A 2-ml column (a 5-ml disposable Costar plastic pipet) of monoclonal antibody Affi-Gel 10 is prepared. The column is washed successively with 3 column volumes of 0.05 *M* phosphate-buffered saline, pH 8.0 (column buffer), 1 *M* acetic acid, and column buffer. Ten milliliters of the preparation from the above purification step is applied to the column. The column is then washed with 10 ml of column buffer followed by 6 ml of 0.01 *M* ammonium acetate, pH 6.5, to remove the salt that is present in the column buffer. The EGF is then stripped from the column with 6 ml of 1 *M* acetic acid and 1-ml fractions are collected.

The pattern of electrophoretic mobility of the two major forms of hEGF together with mouse and rat EGF is shown in Fig. 2. It can be seen that monoclonal antibody affinity chromatography yields a mixture of the two major forms of hEGF in a highly purified state.[11]

The preparation of hEGF from the affinity column is assayed for biological activity by measuring the growth stimulation of human foreskin fibroblasts in culture. At 10 ng/ml hEGF stimulates growth of these cells approximately 2-fold (Table II). At 1 ng/ml a 30% increase in cell growth is observed.

Discussion

The procedure we have described in this chapter permits the isolation of approximately 100 μg of hEGF from 5 liters of raw urine with an average yield of 50%. As shown in Fig. 2, the hEGF elutes from the monoclonal antibody affinity column in a highly purified state as two major forms, hEGF-1 and hEGF-2.[11] These two forms of hEGF can be easily separated by DEAE-cellulose chromatography as described previously.[11] Interestingly, the minor form of hEGF from urine, hEGF-A, found previously utilizing a rabbit antibody affinity column,[15] was not observed in preparations using the monoclonal antibody affinity column. It could be that this minor form of hEGF has a weak binding affinity for

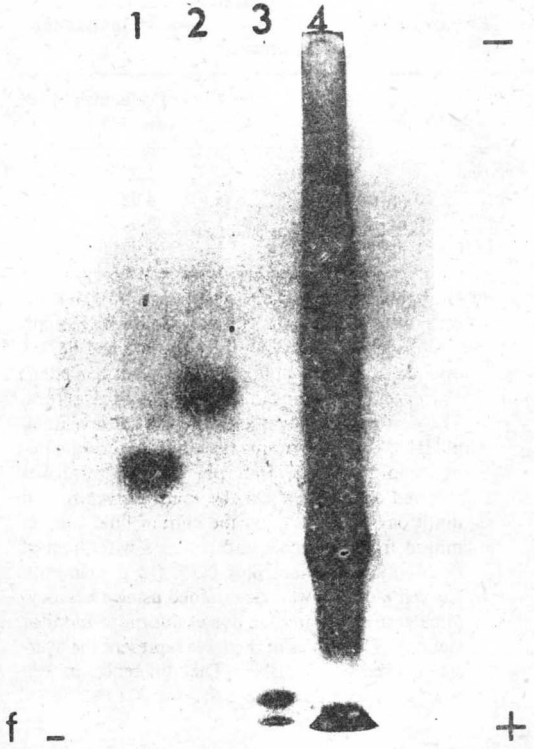

FIG. 2. Polyacrylamide gel electrophoresis of mouse, rat, and human EGF. Polyacrylamide gel electrophoresis was carried out in standard 7.5% slab gels at pH 9.5.[18] The samples were electrophoresed at 25° for 3.5 hr at 20 mA per gel and the gels were subsequently stained for 10 min with Coomassie brilliant blue R250 (0.24% w/v solution in methanol/H_2O/acetic acid, 5/5/1). The gel was destained by soaking in methanol/H_2O/acetic acid, 4/33/3. Lanes 1 and 2 contained 5 μg of mouse EGF and rat EGF, respectively [isolated by the method of R. P. Schaudies and C. R. Savage, Jr., *Comp. Biochem. Physiol.* **84B**, 497 (1986)]; lane 3 contained about 8 μg of a mixture of hEGF-1 (slower migrating) and hEGF-2[11] (faster migrating); and lane 4 contained 20 μl of the hEGF preparation just prior to affinity chromatography. Running dye front is labeled "f."

the monoclonal antibody or the antibody is reactive with an epitope of hEGF-1 and hEGF-2 which is not present on hEGF-A. We believe hEGF-1 is the intact molecule, whereas, hEGF-2 lacks the carboxy-terminal Arg or Leu-Arg residues.[11] The structure of hEGF-A is not known.

[18] B. J. Davies, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).

TABLE II
EFFECT OF EGF ON HUMAN FORESKIN FIBROBLAST GROWTH[a]

Additions	Number of cells/dish × 10^6 (day 11)
None	1.82
mEGF; 10 ng/ml	3.68
hEGF; 10 ng/ml	3.64
hEGF; 1 ng/ml	2.37

[a] Human foreskin fibroblast (passage #5), 4×10^5 cells, were seeded into 100-mm CoStar tissue culture dishes in 5 ml of Dulbecco's modified Eagle's essential medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum. The next day EGF was added to the cultures in 50 μl Hanks' BSS containing 0.2% bovine serum albumin and 25 m*M* HEPES, pH 7.4. Control dishes received only buffer. On the fourth, seventh, and ninth days after seeding, the culture fluid was removed from all dishes and replaced with 5 ml of fresh culture medium plus EGF. On the eleventh day cell numbers were determined using a hemocytometer and trypan blue dye exclusion to monitor viability. The values in the table represent the average between two dishes. The difference in cell counts between duplicate dishes was < 15%.

An attempt was made to use the immobilized monoclonal antibody at the beginning of the purification procedure, thereby hoping to eliminate the first three steps of our purification protocol. Addition of immobilized antibody in a batch-wise fashion directly to neutralized urine with stirring overnight was unsuccessful due to poor absorption of the hEGF to the antibody. Likewise, passage of neutralized urine directly over a monoclonal antibody affinity column was not effective when large volumes (20–40 liters) of urine were used. Thus, we find it necessary to remove large amounts of the insoluble salts, denatured protein, and pigments before using the monoclonal antibody affinity column. Our first three steps consisting of batch Bio-Rex 70, ethanol precipitation, and DE-52 cellulose chromatography are rapid, highly efficient (a 500-fold concentration of hEGF with a > 90% recovery), and can easily be used to work up large volumes of raw urine (40–80 liters).