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PURIFICATION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR BY TYROSINE-SEPHAROSE AFFINITY CHROMATOGRAPHY

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SUMMARY: The EGF receptor has been purified from human epidermoid carcinoma A431 cells by affinity chromatography on wheat germ agglutinin-agarose and tyrosine-Sepharose. The purified EGF receptor was shown to be homogenous by SDS-polyacrylamide gel electrophoresis and possessed EGF-sensitive tyrosine kinase activity. Kinetic analysis of the autophosphorylation indicated that approximately 1.4 mol of phosphate was incorporated per mol of the EGF receptor. When a synthetic tyrosine-containing peptide was used as a phosphorylatable substrate, the specific activity of the EGF-stimulated kinase was 66 nmol/min/mg.

Epidermal growth factor (EGF) is a potent mitogen for a variety of epidermal and epithelial cells in vitro and in vivo (1). The effects of EGF are exerted through binding to a specific membrane-associated receptor of 170,000 dalton (2,3). Like several retroviral transforming proteins, the EGF receptor possesses protein kinase activity which phosphorylates itself and other substrates on tyrosine residue (4-6).

For the characterization of the EGF receptor, several methods of purification have been developed. For example, Cohen et al. utilized a two-step procedure involving EGF-Affigel-affinity chromatography and WGA (wheat germ agglutinin)-Sepharose chromatography (4). Parker et al. (7), Weber et al. (8) and Yarden et al. (9) developed immunoaffinity chromatography using monoclonal antibodies directed against the EGF receptor.

In the present report, we describe the purification of the EGF receptor from human epidermoid carcinoma A431 cells, using tyrosine-Sepharose-affinity chromatography, which has been successfully utilized

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for the purification of tyrosine-O-phosphate phosphatase and $pp60^{src}$ by Fukami and Lipmann (10-12). The highly purified active EGF receptor can be obtained by this rapid and convenient procedure.

MATERIALS AND METHODS

Cells: Human epidermoid carcinoma A431 cells (13) were grown in Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum.

Membranes: A431 cells were washed with PBS and scraped with rubber policeman. The cells collected by centrifugation at 1,200 rpm at 4°C were homogenized (20 strokes) in hypotonic KMP buffer (20 mM Pipes (1,4-piperazine-diethanesulphonic acid)-NaOH pH 7.2, 1 mM MgCl, 5 mM KCl). The homogenate was centrifuged at 15,000 × g for 5 min at 4°C. The pellet was resuspended in KMP buffer and centrifuged again. The two supernatants were combined and centrifuged at 100,000 × g for 30 min. The pellets were used as a membrane fraction.

Purification of the EGF receptor: Membrane fractions prepared from A431 cells were solubilized by addition of Triton X-100 to a final concentration of 1%. After incubating for 20 min at 0°C, the insoluble material was removed by centrifugation at 100,000 × g for 30 min. The supernatant was incubated with WGA-agarose (Pharmacia) for 1 h at 4°C. The gel was poured into a column and washed with buffer A (40 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)-NaOH pH 7.2, 0.5 M NaCl, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 10% glycerol, 0.05% Triton X-100). The EGF receptor was eluted with buffer A containing 0.3 M N-acetylglucosamine. The active fractions were diluted with the same volume of buffer B (40 mM Hepes-NaOH pH 7.2, 1 mM dithiothreitol, 0.2 mM PMSF, 10% glycerol, 0.05% Triton X-100) containing 1.6 M $(NH_4)_2SO_4$ and applied to a tyrosine-Sepharose column equilibrated with buffer B containing 0.8 M $(NH_4)_2SO_4$. After washing with the same buffer, the EGF receptor was eluted with buffer B containing 0.25 M $(NH_4)_2SO_4$. The fractions containing kinase activity were pooled and then dialyzed against buffer B. The dialyzed fraction was applied to the second tyrosine-Sepharose column equilibrated with buffer B. The EGF receptor was eluted with buffer B containing 0.25 M $(NH_4)_2SO_4$, dialyzed against buffer B and stored at -80°C. Tyrosine-Sepharose was prepared by coupling tyrosine to activated CH-Sepharose 4B (Pharmacia).

Assay of protein kinase activity: Protein kinase reactions were carried out in a final volume of 50 µl containing 20 mM Hepes-NaOH pH 7.2, 10 mM MgCl, 3 mM MnCl, 1 mM dithiothreitol, 20 µM (γ-^{32}P)ATP (4 mCi/µmol), 100 ng/ml mouse EGF (Collaborative Research), and the purified EGF receptor (100 ng). The EGF receptor was first incubated with EGF for 30 min at 27°C before assay of kinase activity. The kinase reactions were initiated by the addition of (γ-^{32}P)ATP and continued for 5 min at 30°C. The reactions were stopped by addition of Laemmli's SDS buffer (14) and boiling for 2 min. The samples were analyzed by SDS polyacrylamide gel electrophoresis (14) and autoradiography. The bands of the EGF receptor were excised from the gels and the radioactivity was counted with a liquid scintillation counter.

When the src-related synthetic peptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, (final 4 mM) (Peninsula Laboratory) was used as a substrate, the reaction was terminated by addition of 25 µl of 10% trichloroacetic acid and 6 µl of bovine serum albumin (10 mg/ml) (15). Precipitated proteins were removed by centrifugation and 60 µl aliquot of the supernatant was spotted on a square

of phosphocellulose paper. The papers were washed in acetic acid (16) and the radioactivity was counted with a liquid scintillation counter.

RESULTS AND DISCUSSION

We used detergent solubilized A431 cell membranes as a source for the purification of the EGF receptor. The Triton extract of A431 cell membranes was first absorbed on the WGA-agarose column and specifically eluted with 0.3 M N-acetylglucosamine. Further purification was performed by the tyrosine-Sepharose chromatography, based on the observation that the EGF receptor binds to the tyrosine-Sepharose at a salt concentration of either > 0.55 M or < 0.05 M in $(NH_4)_2SO_4$. At first the material eluted from WGA-agarose column was applied to the tyrosine-Sepharose column which had been equilibrated with 0.8 M $(NH_4)_2SO_4$. The EGF receptor was eluted from the column by lowering the concentration of $(NH_4)_2SO_4$ to 0.25 M and then subjected to the second tyrosine-Sepharose chromatography at low ionic strength (in the absence of $(NH_4)_2SO_4$). From this column, after thorough washing, the highly purified EGF receptor could be eluted by 0.25 M $(NH_4)_2SO_4$. A summary of the purification is presented in Table 1.

The EGF receptor purified by this method was shown to be homogenous by SDS polyacrylamide gel electrophoresis (Fig 1A) and possessed the EGF sensitive autophosphorylation activity when incubated with $(\gamma\text{-}^{32}P)ATP$

Table 1. Purification of the EGF receptor

Fraction	Protein, (mg)	Specific activity, (nmol/min/mg)[1]	Total activity, (nmol)[1]	Recovery, %	Purification factor
Membrane	500	0.04	20	100	1
WGA-agarose	28.5	0.6	17.1	86	15
Tyrosine-Sepharose 1	0.35	24.6	8.61	43	615
Tyrosine-Sepharose 2	0.11	34.4	3.80	19	860

[1]The EGF receptor kinase activity was assayed with the tyrosine-containing synthetic peptide at 0°C for 3 min.

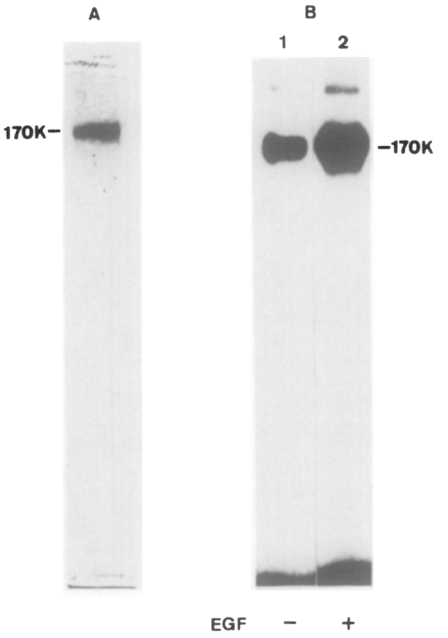

Fig. 1. SDS-polyacrylamide gel electrophoresis of the purified EGF receptor. (A) The EGF receptor was purified from detergent extract of A431 cell membranes by affinity chromatography on WGA-agarose and tyrosine-Sepharose as described under "MATERIALS AND METHODS". The purified receptor was electrophoresed through a 8% polyacrylamide gel and stained with silver. (B) The purified receptor was phosphorylated in vitro with (γ-^{32}P)ATP for 5 min at 30°C in the absence (lane 1) and presence (lane 2) of EGF and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

(Fig. 1B). The time course of the autophosphorylation in the presence and absence of EGF is shown in Fig. 2. EGF stimulated the autophosphorylation approximately 2-fold. In the presence of EGF, approximately 1.4 mol of ^{32}P was incorporated / mol of the receptor. Fig. 3 shows the time course of the phosphorylation of a synthetic peptide containing the autophosphorylation site of pp60^{src} by the purified EGF receptor. The phosphorylation reaction continued to proceed over 30 min. EGF stimulated a 5-fold increase in the incorporation of ^{32}P into the synthetic peptide. Fig. 4 shows the Lineweaver-Burk plot for the phosphorylation of the synthetic peptide by the purified EGF receptor. The K_m for the peptide substrate was 2 mM and the V_{max} was 66 nmol/min/mg in the presence of EGF.

The EGF receptor purified by EGF-Affigel-affinity chromatography was previously reported to incorporate approximately 0.3 to 0.4 mol of

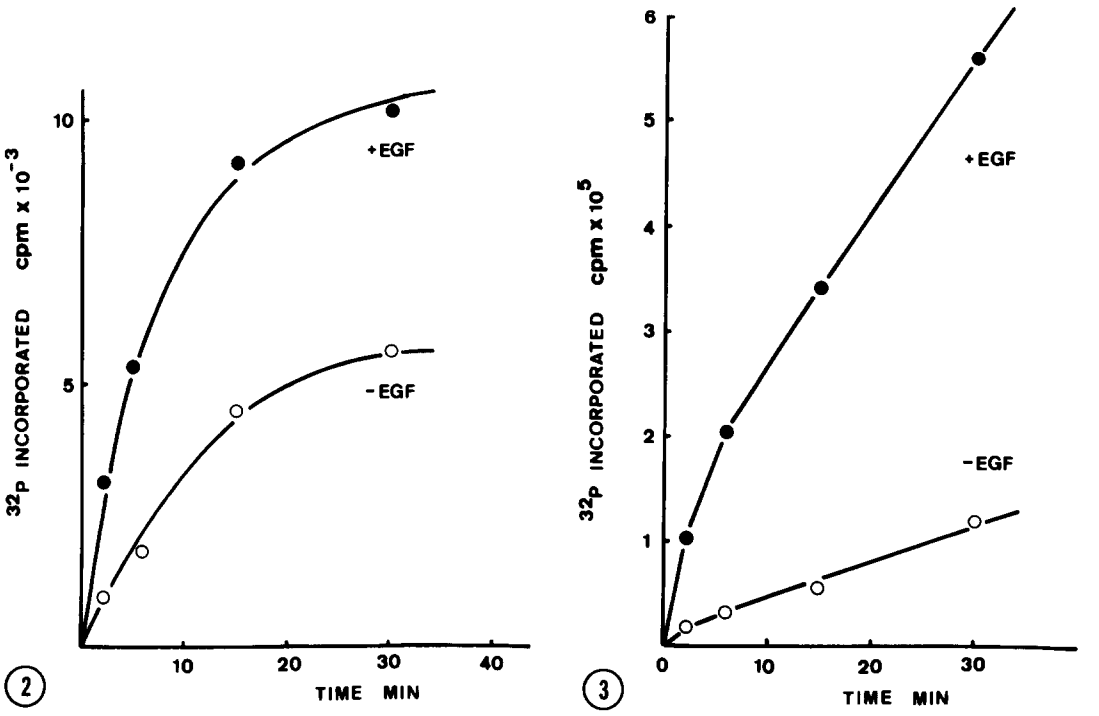

Fig. 2. Time dependence of the autophosphorylation of the purified EGF receptor. The purified EGF receptor was assayed for autophosphorylating activity at 30°C (see "MATERIALS AND METHODS") in the absence (O) or presence (●) of EGF for the indicated time periods.

Fig. 3. Time course of the phosphorylation of the tyrosine-containing synthetic peptide by the purified EGF receptor. The synthetic peptide (4 mM) was phosphorylated in the same assay mixture as used for autophosphorylation in the absence (O) or presence (●) of EGF. The reaction products were analyzed as described under "MATERIALS AND METHODS".

phosphate / mol of the receptor molecule (17) and to have a V_{max} of 15.7 nmol/min/mg when a synthetic peptide was used as substrate (18). Therefore, the purified EGF receptor reported here is considered to be less denatured and have significantly higher protein kinase activity than that provided by EGF-Affigel chromatography.

Recently, immunoaffinity purification utilizing the monoclonal antibodies directed against the EGF receptor has been developed by several investigators (7-9). However, immunoaffinity purification can be achieved only when the appropriate monoclonal antibody is available. On the contrary, the procedure described here is attainable without any special materials.

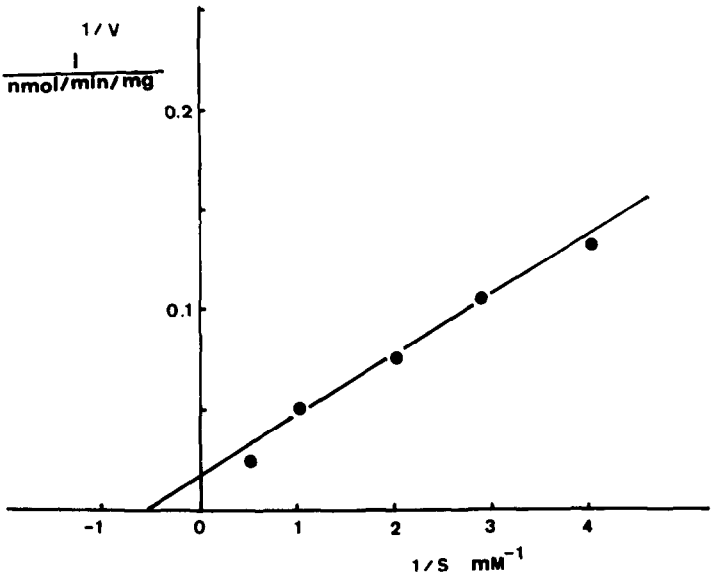

Fig. 4. Lineweaver-Burk plot of the phosphorylation of the synthetic peptide by the purified EGF receptor. The initial velocity of phosphorylation of the synthetic peptide was estimated during a 3 min incubation in the presence of EGF as described under "MATERIALS AND METHODS".

In summary, the procedure described here provides an effective, simple and economical means of obtaining a highly purified active EGF receptor. Furthermore, tyrosine-Sepharose affinity chromatography will be also useful for the purification of the other tyrosine-specific protein kinases.

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