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EPIDERMAL GROWTH FACTOR (EGF) BINDING TO MEMBRANES IMMOBILIZED IN MICROTITER WELLS AND ESTIMATION OF EGF-RELATED TRANSFORMING GROWTH FACTOR ACTIVITY

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The binding of radiolabeled epidermal growth factor (EGF) to immobilized A-431 target cell membranes coupled to polyvinyl chloride microtiter wells is described. Saturation curves and Scatchard analysis of the data indicate that the observed binding parameters are consistent with those previously reported. Binding capacity of the membranes are approx. 6.6 pmol EGF per mg membrane protein. Kinetics of ^{125}I-EGF binding were slower, however, than reported for binding to membranes in suspension, although binding constants were not greatly different. The high- and low-affinity binding constants for ^{125}I-EGF were calculated to be approximately $1 \cdot 10^{12}$ M^{-1} and $2.5 \cdot 10^{9}$ M^{-1}, respectively. Application of this technique in a competitive binding assay requires no more than 2.5 μg of membrane protein per assay, is essentially complete after 60 min, and facilitates screening of a large number of samples in a short time. Therefore, this will assist in the evaluation and quantitation of EGF and EGF-related transforming growth factor activity in physiological fluids. This technique may also be applied to analyses of other hormone-receptor systems.

Introduction

Our laboratory has been studying transforming growth factor-like activity in human physiological fluids. Transforming growth factors are peptides in whose presence normal cells reversibly acquire characteristics of a transformed phenotype which include anchorage-independent growth and loss of contact inhibition [1,2]. Transforming growth factors (TGFs) are produced by a variety of virally and chemically transformed tissue culture cell lines [1,3–5] as well as solid tumors [5,6] and are also found in body fluids such a serum [7] and urine (Refs. 8 and 9; Kimball, E.S. et al., unpublished data). Furthermore, some transforming growth factors are functionally and structurally related to epidermal growth factor (EGF), can bind to EGF receptors, and are characterized according to their ability to compete for binding to EGF-receptors. Thus, α-TGFs bind to EGF receptors and promote in vitro colony formation whereas β-TGFs are incapable of competing for EGF receptors and must act synergistically with EGF or α-TGFs in order to induce colony formation in vitro [5,10,11]. One means of separating and comparing these various transforming growth factors is by high-performance liquid chromatography (HPLC). However, most EGF competitive binding assays in current use either do not lend themselves well to screening large numbers of samples from HPLC and other types of chromatography, or otherwise require a time-consuming and not always efficient

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Abbreviations: EGF, epidermal growth factor; HPLC, high-performance liquid chromatography.

method for solubilizing and quantitating bound radioactivity [2,5].

A more rapid competitive binding assay for EGF and EGF-related transforming growth factors was developed to overcome the foregoing. This assay takes advantage of the fact that isolated membranes of target cells bearing EGF receptors are often as efficient in binding EGF as are freshly grown and/or formalin-fixed target cells [2,12,13]. By binding these membranes to 96-well poly(vinyl chloride) plates, the assay can readily be used for screening large numbers of samples. The binding parameters of EGF to immobilized membranes have not previously been studied and we present these data, as well as demonstrate the applicability to screening reverse-phase HPLC eluates of normal urine extracts for EGF-related transforming growth factors.

Materials and Methods

Materials. HPLC solvents were purchased from Burdick and Jackson Chemicals (Muskegon, MI). Trifluoroacetic acid was obtained from Pierce Chemicals (Rockford, IL). Commercial mouse EGF was purchased from Collaborative Research, Inc. (Waltham, MA). Poly(vinyl chloride) plates were purchased from Dynatch (Arlington, VA). Ultrafiltration membranes and apparatus were purchased from Amicon Corp. (Danvers, MA).

HPLC of EGF and transforming growth factors. An Altex model 324 HPLC (Beckman Instruments, Palo Alto, CA) was used to further purify commercial EGF and to help separate urinary proteins prior to testing for EGF-competing activity. An Hitachi variable wavelength HPLC ultraviolet light detector was used to monitor column effluents and an LKB model 2111 fraction collector was used to collect HPLC fractions (LKB Instruments, Rockville, MD). A Waters (Waters Associates, Milford, MA) C-18 μ-Bondapak ® reverse-phase HPLC column (250 mm $\times$ 3.9 mm) was used throughout these studies.

Commercial mouse EGF was further purified using a linear gradient of 0 to 20% acetonitrile in 0.05% trifluoroacetic acid, pH 2.5, over a 5 min period and then to 60% acetonitrile, 0.05% trifluoroacetic acid, pH 2.5 over a period of 60 min at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected. Fractions containing α-EGF (see below) were pooled and used in subsequent experiments.

Transforming growth factors in urine were resolved from one another using a linear gradient from 0.05% trifluoroacetic acid, pH 2.5 to 0.05% trifluoroacetic acid/60% acetonitrile over a period of 120 min. A flow rate of 1 ml/min was used and 1 ml fractions were collected.

Preparation of urine for transforming growth factor analysis. Urine from normal donors was acidified to 1 M with acetic acid, centrifuged for 30 min at 20000 $\times$ g and passed through a 0.45 μm filter. 10 mg of protein in the urine filtrate was concentrated by ultrafiltration on an Amicon XM-50 membrane and the retentate so obtained, containing approximately 0.4–0.5 mg protein, was applied to the HPLC column. Ten percent of each column fraction was assayed for EGF-competing activity. Soft agar growth promoting activity was determined as previously described [5].

Protein determinations were performed using a Bio-Rad ® colorimetric assay [14] and the method of Lowry et al. [15]. Because it was difficult to standardize urinary protein determinations against commercial protein standards, dialyzed ($M_r \geqslant$ 100), lyopholized urine protein was used as a standard.

EGF binding assay. EGF, isolated as above, was radioiodinated with ^{125}I (Amersham, Arlington Heights, IL) as described [16] to a specific activity of 21.5 μCi/μg of protein. A-431 human epidermoid carcinoma cells, which have a high density of EGF receptors [17], were grown to confluency in roller bottles. Membranes were subsequently obtained by hypotonic lysis using the method of Thom et al. [18] and dispensed into 96-well poly(vinylchloride) plates at 2.5 μg membrane protein/well in 100 μl Dulbecco's phosphate-buffered saline. The plates were dried overnight at 37°C, sealed and then stored at 4°C until required. Prior to use, the dried cell membranes were washed 4 times with 150 μl binding buffer (Dulbecco's Modified Eagles Medium containing 1 mg of bovine serum albumin per ml and 50 mM 2-[bis(2-hydroxyethyl)amino]ethane, pH 6.8) and then pre-incubated 30 min at 23°C with 150 μl of binding buffer. Binding was initiated by the addition of 100 μl of binding buffer containing 4 ng/ml of ^{125}I-EGF with or without inhibitor. The mixtures

were incubated with the immobilized membranes for 1 h at 23°C and then removed by aspiration. Wells were washed four times with 200 μl of fresh binding buffer, separated from the plate with a hot wire and deposited into test tubes to determine bound ^{125}I-EGF. Nonspecific binding, determined by using a 100-fold excess of unlabeled commercial grade mouse EGF, was subtracted to obtain specific ^{125}I-EGF binding.

Percent inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = 100 - ([\text{cpm bound (unknown)} - \text{nonspecific cpm bound}] / [\text{cpm bound (no inhibitor added)} - \text{nonspecific cpm bound}]) \times 100$$

EGF radiobinding assays using intact formalin-fixed cells were performed in 24-well or 96-well cluster plates (Costar, Cambridge, MA) according to the method of DeLarco et al. [2].

Results

Fig. 1 shows the HPLC elution profile of commercial mouse EGF. Two peaks of EGF activity were obtained. The major peak (α) eluted at 39.5% acetonitrile and contained 38 μg of protein or 76% of the original sample. The minor peak with EGF activity (β) eluted at 50% acetonitrile and contained approx. 2 μg of protein. Two chromatographically distinct forms of EGF have also been reported by other investigators using HPLC [19,20]. The major form (α) of EGF has been reported to be stable to freezing and thawing, whereas β-EGF is not [20]. Accordingly, only α-EGF was used in subsequent experiments.

Fig. 2 depicts the binding at 23°C of ^{125}I-EGF to increasing amounts of A-431 membrane protein, presented as immobilized membranes or to membranes treated in suspension and collected by filtration through glass fiber filters [12]. These results demonstrate that both preparations of membrane fragments bound equivalent amounts of ^{125}I-EGF, but there was a significantly higher background when the latter procedure was examined. No more than 10 μg of immobilized membrane protein per well was used because previous studies in our laboratory showed that that is the upper limit of protein which can be bound to the wells.

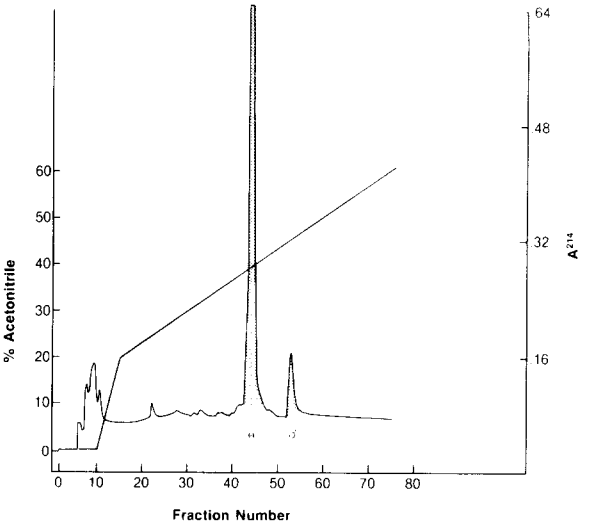

Fig. 1. Reverse-phase HPLC of commercial EGF. See Materials and Methods for details. Shaded areas correspond to fractions containing EGF activity which was determined using the method of DeLarco et al. [2]. Protein was monitored at 214 nm.

The kinetics of ^{125}I-EGF binding to immobilized membranes was studied at three different temperatures: 4, 23 and 37°C (Fig. 3). The greatest binding occurred at 37° and the least binding at 4°C, as previously observed by other investigators

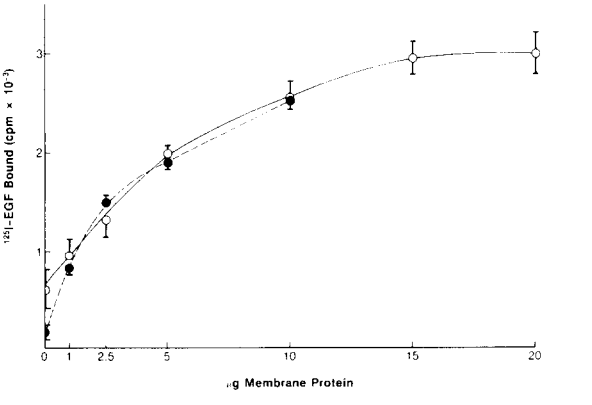

Fig. 2. Comparison of binding of ^{125}I-EGF to A-431 membrane fragments. Increasing doses of membrane protein bound to poly(vinyl chloride) wells (●) were compared to membranes treated in suspension (○). Binding of 0.4 ng ^{125}I-EGF to the membranes was carried out at 23°C for 1 h.

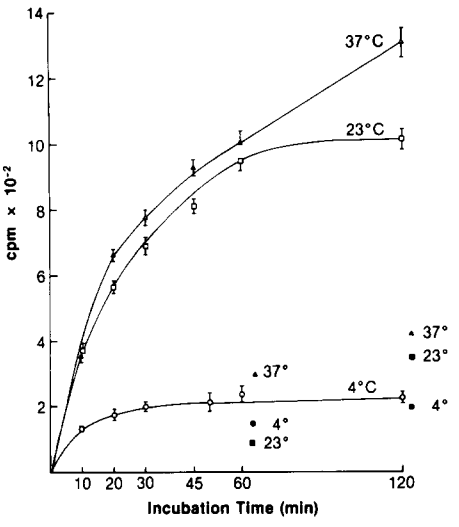

Fig. 3. Kinetics of ^{125}I-EGF binding (0.4 ng protein) to 2.5 μg A-431 membranes. Three different temperatures (4°C, 23°C and 37°C) were studied for the times indicated. Curves are corrected for nonspecific binding, using a 100-fold excess of unlabeled EGF. Closed symbols, indicating nonspecific binding at 60 min and 120 min, are shown in the figure.

[12–14]. However, nonspecific binding was unacceptably high at 37°C and when the binding curves were corrected accordingly, the difference between binding at 23 and 37°C was noticeably less. For this reason 23°C was selected as the optimal temperature in subsequent assays. Unlike earlier studies with isolated A-431 membranes [2,12], maximum binding was not achieved within 15 min. Instead, at least 60 min incubation was required. Equilibrium was reached within 120 min, but nonspecific binding was greatly increased for incubation periods longer than 60 min.

A series of competitive binding curves using different doses of A-431 membranes and 0.4 ng ^{125}I-EGF/well are shown in Fig. 4. The standard curve obtained from 2.5 μg/well provided the most sensitive curve for our purposes, particularly because it was linear between 0.1 ng and 1 ng of EGF inhibitor. As shown, these curves were comparable to those obtained using 10^4 A-431 cells grown in microwells and formalin-fixed.

The binding of ^{125}I-EGF to A-431 membrane fragments was saturable, as shown in Fig. 5A. The data indicate that 6.6 pmol ^{125}I-EGF were bound by 1 mg membrane protein, similar to the results

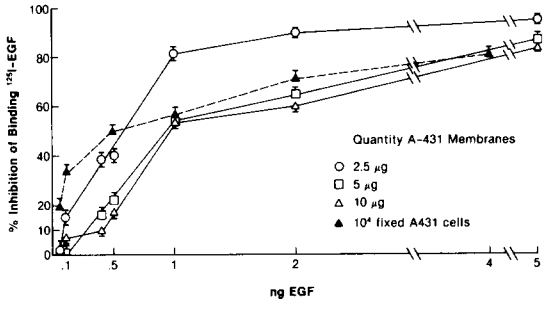

Fig. 4. Inhibition of ^{125}I-EGF binding by EGF. Increasing amounts of unlabeled EGF were titrated vs. 0.4 ng ^{125}I-EGF. Binding at these different doses of membrane protein (2.5 μg, 5 μg and 10 μg per well) were compared with binding to the standard number ($1 \cdot 10^4$) fresh formalin-fixed A-431 cells in a 96-well Costar tissue culture dish.

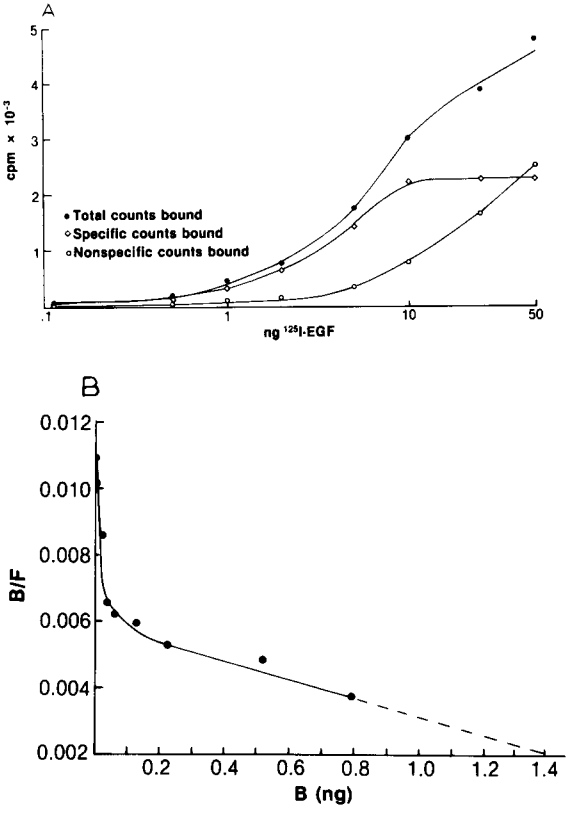

Fig. 5. (A) Saturation curve of ^{125}I-EGF binding to 1 μg A-431 membrane protein. Curves show total binding, nonspecific binding with excess unlabeled EGF and specific binding. Incubation period was for 2 h at 23°C. (B) Analysis of binding data according to Scatchard [21].

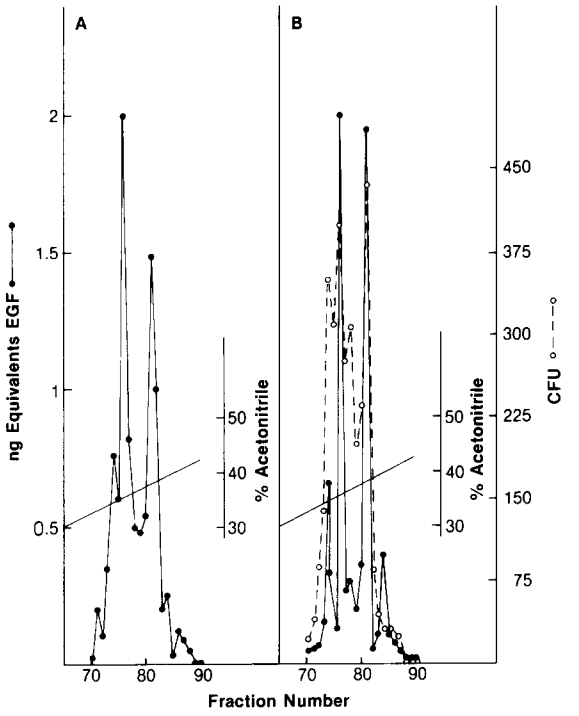

Fig. 6. Reverse-phase HPLC of acid extract of normal urine. EGF competing activity in fractions is shown for (A) solid-phase membrane assay using 0.4 ng ^{125}I-EGF and 2.5 μg A-431 membranes and (B) 0.4 ng ^{125}I-EGF and $2.5 \cdot 10^4$ formalin-fixed A-431 cells in a 24-well tissue culture dish. Transforming growth factor activity, in colony forming units (CFU), is indicated by the broken line in panel B only.

reported by Carpenter et al. [12]. Scatchard analysis [21,22] of these data (see Fig. 5B) revealed binding heterogeneity, suggesting receptors with a range of affinities for EGF. This is in agreement with results reported by other investigators [2,12,20] and binding constants similar to those previously published [2,12,13,19] were observed in this study. Thus, in the high-affinity region of the curve the association constant was estimated to be approx. $1 \cdot 10^{12}$ M^{-1} whereas the low-affinity association constant was estimated to be about $2.5 \cdot 10^9$ M^{-1}.

Finally, reverse-phase HPLC of an acid extract of normal donor urine was used for an examination of transforming growth factors with EGF-competing activity. Fig. 6 demonstrates that the present solid-phase assay is as sensitive as the standard EGF radioreceptor binding assay with intact target cells [2,5]. Both assays were able to detect the same multiple forms of EGF-competing activity with similar degrees of sensitivity. In addition, EGF receptor-binding activity co-chromatographed with transforming growth factor activity.

Discussion

A solid phase EGF binding assay has been developed in which isolated membrane fragments from the A-431 epidermoid carcinoma cell line were immobilized in a 96-well poly(vinyl chloride) plate. Binding of soluble proteins and membrane fragments to poly(vinyl chloride) plates has previously been applied to enzyme-linked immunosorbent assay systems [23]. The present study examines the binding parameters of EGF to immobilized membranes and demonstrates the applicability of this method of receptor immobilization, specifically to quantitating EGF and EGF-related peptides. We suggest that this technique may also be valuable to hormone assays in general. The assay takes advantage of the potential for large-scale screening provided by a 96-well system and is as sensitive as previously reported assays using freshly grown or formalin-fixed cells [2,5] or membrane fragments held in suspension [12], but has fewer complications and greater speed than the latter. Assay systems which use membranes held in suspension are limited by the capacity of the filtration manifold and by slow filtration rates which often occur because of clogged filters or loss of vacuum. We also noted high background levels where there was incomplete washing of the manifold between sample applications, and cross-contamination resulting therefrom. The use of formalin-fixed cells, which is the standard assay [2] does not lend itself well to rapid, high volume screening unless the cells are grown in 96-well plates. In our hands, this approach often suffered from batch-to-batch variation of cells plated out as targets and requires a time-consuming harvesting step. Advantages of the solid-phase assay system include the ease of preparing target plates, rapidity of the assay and the ability to use a single stable source of target receptors over a long period of time. In addition, membranes may be plated out as long as 4 weeks in advance with no noticeable effect on binding capacity (data not shown).

Binding of ^{125}I-EGF was shown to be saturable, but the kinetics of binding in the solid phase were

considerably slower than that seen when membrane fragments were treated in suspension [12]. Scatchard analysis [21,22] of the binding data was in agreement with previously published binding constants [2,12,13,20], but also suggests that there may be fewer high-affinity sites available. This may be a reflection of the particular A-431 preparation used in this study, as opposed to differences in physical states between solid-phase membrane fragments and those in suspension, but nevertheless is consistent with the slower binding kinetics observed. In either case, the sensitivity and reproducibility of the assay was in no way adversely affected when compared to previously established methodology.

As a means of verifying the efficacy of this solid-phase assay, EGF-competing activity in HPLC fractions containing transforming growth factor activity was examined. Multiple transforming growth factors were detected between 32% and 38% acetonitrile. Quantitation of EGF-receptor binding material showed concordance between the solid-phase assay and the standard assay [2]. The solid-phase assay could also detect small amounts of EGF-competing activity not readily detected otherwise. It is important to note that the source of the transforming growth factor activity was an acid extract of urine from a normal donor. The presence of these factors in physiological fluids of normal individuals is under further study and is being compared to transforming growth factors obtained from patients with disseminated cancer as well as other disease. However, the occurrence of transforming growth factors in normal urine poses a number of questions regarding the physiological role of transforming growth factors in normal metabolic processes vs. neoplasia. Also, the relationship between urinary transforming growth factors (Refs. 8 and 9; Kimball, E.S. et al., unpublished data) and tumor-derived transforming growth factors [1,3–6] is of obvious interest and demands further study. Such examinations will be facilitated by the solid-phase EGF competitive binding assay presented here.

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