Journal of Supramolecular Structure 14:441–459 (1980)
Control of Cellular Division and Development: Part A: 283–301

Direct Linkage of EGF to Its Receptor: Characterization and Biological Relevance

Peter S. Linsley and C. Fred Fox

Department of Microbiology and Parvin Cancer Research Laboratories, Molecular Biology Institute, University of California, Los Angeles, California 90024

A small portion of the ^{125}I-EGF that binds specifically to intact cells or isolated membranes from a variety of sources becomes directly and irreversibly linked to EGF receptors. This provides a simple technique for affinity labeling the EGF receptor. Membranes isolated from the human epidermoid carcinoma cell line A431, which posesses extraordinarily high numbers of EGF receptors, gave rise to three major direct linkage complexes of MW = 160,000, 145,000, and 115,000. The time course for formation of each is similar, showing that ^{125}I-EGF can form direct linkage complexes with several preexisting forms of the EGF receptor. The direct linkage of EGF to receptor is slow in comparison to ^{125}I-EGF binding, but both processes have similar susceptibilities to competition by unlabeled EGF.

EGF was modified chemically with the amino site-specific reagent, N-hydroxysuccinimidyl biotin. The biotinyl-EGF had a reduced capacity to engage in direct linkage complex formation with no concomitant reduction in its ability to bind to EGF receptors. Since native and biotinyl EGF have identical abilities to stimulate the uptake of ^{3}H-thymidine into DNA when incubated with cultured murine 3T3 cells, the direct linkage of EGF to its receptor does not appear to play an important role in EGF-stimulated mitogenesis.

Key words: EGF receptors, biotinyl EGF, covalent EGF-receptor complexes – and 3T3 cell growth regulation, on human placental membranes, on cultured cells

Epidermal growth factor (EGF) is a potent polypeptide mitogen that initiates its biological activity by interacting with the cell surface [1]. Radioiodinated derivities of EGF bind to specific high-affinity receptors on responsive cells [2–6]. Rapid internalization and lysosomal degradation of EGF follow binding and have been demonstrated by a

Abbreviations: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle medium; ST buffer, 0.25 M sucrose in 10 mM Tris·HCl at pH 7.4; PBS, 0.15 M NaCl in 0.1 M sodium phosphate at pH 7.4; DBH, DMEM minus $NaHCO_3$, but containing 0.1% bovine serum albumin in 10 mM HEPES buffer at pH 7.4; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; ESB, electrophoresis sample buffer (63 mM Tris·HCl at pH 6.8, 3% SDS, and 10% glycerol); NHS Biotin, N-hydroxysuccinimidyl biotin; MW, molecular weight (M_r).

Received August 25, 1980; accepted October 3, 1980.

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variety of biochemical and optical techniques [5, 7–11]. Because the process of down regulation (ie, EGF-mediated loss of its own binding capacity) occurs concomitantly with the internalization and degradation of receptor-associated EGF in the lysosomes, the EGF receptor was presumed to be processed similarly [7]. In this laboratory, we have investigated directly the fate of receptor after EGF addition. Photoaffinity probes, which crosslinked radioiodinated EGF to its receptor, were used initially for receptor identification [12]. The photoaffinity crosslinked EGF–receptor complex, like EGF, was internalized and degraded in the lysosomes [13]. Other strategies for identifying EGF receptors by photoaffinity labeling have been reported [14].

Recently we [15] and Baker et al [16] independantly observed that a small portion of the radiolabeled EGF that binds specifically to murine 3T3 cells or human foreskin fibroblasts becomes directly and irreversibly linked to EGF receptors. The EGF–EGF receptor direct linkage complex has properties identical to those of the EGF–EGF receptor complex formed by photoaffinity labeling. Thrombin may also undergo direct linkage to its receptor during mitogenic stimulation [16].

Direct linkage affords an obvious technical advantage since photoaffinity probes, which are difficult to synthesize and use, need not be employed to specifically label EGF receptors. While the biological role of direct linkage was not obvious, we were intrigued by the possibility that it might be physiologically important, perhaps representing the small fraction of "very high affinity binding sites," which Schechter et al [17] proposed to mediate the biological effects of EGF.

Because of its technical utility and potential biological importance, we have investigated the direct linkage process in detail, but we find no evidence for its involvement in EGF-induced mitogenesis. In a companion paper [18], we use this technique to document the striking protease sensitivity of EGF receptors in isolated membranes and the specific EGF-stimulated phosphorylation [19] of two fragments of the receptor.

EXPERIMENTAL PROCEDURES

Materials

Male mouse submaxillary glands were from Pel-Freeze. Swiss 3T3 cells (clone 42) were obtained initially from G. Todaro, National Cancer Institute; HF-15 cells (human foreskin fibroblasts), from D. Cunningham, University of California, Irvine; and A431 cells, from G. Todaro and J. De Larco. Iodoacetic acid, biotin and p-nitrophenyl-N-acetyl-β-D glucosaminide were from Sigma. TPCK-treated trypsin (254 units/mg) was obtained from Worthington. Ficoll and Sephadex were obtained from Pharmacia; Iodine-125, from Amersham; and ^{3}H-thymidine, from New England Nuclear.

Cell Culture

Cultures were maintained in DMEM plus 10% fetal calf serum. Cells were grown on plastic dishes and plastic or glass roller bottles in an atmosphere of 10–15% CO_2.

Membrane Preparations

Three procedures were used; all result in substantial enrichment of ^{125}I-EGF binding over that present in crude homogenates.

Procedure I. For preliminary experiments, a crude particulate fraction was prepared after cells were homogenized in hypotonic solution known to disrupt osmotically fragile lysosomes [20]. Cells grown on plastic dishes were washed free of serum and harvested by

scraping into DMEM. The cell pellet was collected by sedimentation at 750g for 5 min, suspended in 10 volumes of 10 mM Tris·HCl 7.4, containing 2 mM phenylmethyl sulfonyl fluoride; the suspension was incubated at 20°C for 10 min. Cells were disrupted by vigorous mechanical homogenization (35 strokes in a hand-driven Dounce B homogenizer), and unbroken cells and nuclei were collected by sedimentation at 750g for 5 min. The supernatant fraction was layered over a cushion of ST buffer. After sedimentation at 24,000g for 30 min, the crude membrane pellet was suspended in ST buffer. These membranes were stored at −20°C for several days with no change in EGF binding activity.

Procedure II. This membrane preparation was utilized in all experiments not specifying procedures I or III. Cells were grown to confluence in roller bottles, growth medium was removed, and the monolayers were washed with 10 mM Tris·HCl, pH 7.4, containing 0.15 M NaCl, and scraped into ST buffer. Cell pellets were collected by centrifugation at 2,000g for 5 min and suspended in 9 volumes of ST buffer. The suspension was homogenized with a tight-fitting, motor-driven Teflon pestle (20 strokes). Nuclei and unbroken cells were removed by centrifugation at 2,600g for 5 min. The particulate matter in the postnuclear supernatant was sedimented at 35,000g for 30 min, and the pellet was suspended in 4 times the original packed cell volume in 35% Ficoll in ST buffer. Ten milliliters of this suspension were overlaid with 10 ml each of 25% and 12% Ficoll in ST buffer. A final overlay of 6 ml of ST buffer was added, and the tubes were sealed and centrifuged for 1.5 h at 167,000g in a Beckman VTi 50 verticle rotor. The material at the 0/12%, 12/25%, and 25/35% Ficoll interfaces was individually isolated, diluted with 10 mM Tris·HCl, pH 7.4, and pelleted at 35,000g for 30 min. The pellets were suspended in a small volume of ST buffer and diluted prior to use. For convenience, membranes were routinely stored at −20°C for periods of several months with little effect on ^{125}I-EGF binding. In Table A-I (see Appendix) the characterizations of two representative preparations are described with respect to ^{125}I-EGF binding, a plasmalemmal marker, and the activity of a lysosomal marker, N-acetyl-β-D-glucosominidase [21]. Material isolated at the 0/12% Ficoll interface contained the highest ^{125}I-EGF binding activity relative to the lysosomal marker enzyme activity; this fraction was the source of membranes used in all experiments unless another procedure is specifically cited.

Procedure III. In some experiments, membranes were prepared by a published procedure [22] in which cells were lysed by dilution into hypotonic buffer prior to a series of differential centrifugations. Membranes prepared by this procedure had a ^{125}I-EGF specific binding activity of 41 pmoles/mg and a specific activity of 0.96 A_{400} units (Table A-1), for N-acetyl-β-D-glucosaminidase.

Preparation and Iodination of EGF

EGF was purified by the method of Savage and Cohen [23] and radioiodinated using chloromine T [12]. The ^{125}I-EGF preparations used in these experiments had specific activities ranging from 6–35 $\times$ 10^8 cpm/nmole.

EGF Binding and Direct Linkage Complex Formation

The procedures used for measuring ^{125}I-EGF binding have been described [12]. Unless indicated otherwise, direct linkage complexes were formed with either intact cells or isolated membranes during a 1 h binding assay at 20°C. In studies with intact cells, the cultures were washed thoroughly with DBH and incubated with the desired concentration of ^{125}I-EGF in DBH. Unbound ^{125}I-EGF was removed by washing the cell monolayers with DBH, and cell-bound radioactivity was determined after dispersion of the cells in 0.5 N NaOH. Total EGF binding was corrected for nonspecific binding measured under identical

conditions, but in the presence of a large excess of unlabeled EGF. When direct linkage was assessed, the final washes were done with protein-free medium to remove BSA (which interfered with gel electrophoresis), and the cell monolayers were solubilized in ESB. In studies with isolated membranes, the reactants were mixed in a total reaction volume of 0.1 ml; the final concentration of BSA in the assay was at least 0.01%. When binding alone was measured, membrane-bound ^{125}I-EGF was separated from the mixture by filtration on Whatman GFC filters, and the filters were washed thoroughly with DBH. For direct linkage measurements, membranes were sedimented in a Brinkman microfuge through a cushion of 10% sucrose in DBH (15 min at 12,800g) to remove unbound ^{125}I-EGF. The membrane pellet was given a final wash with BSA-free medium prior to addition of ESB. Nonspecific binding was measured in the presence of 2.4 μM unlabeled EGF. Roughly equivalent binding affinities were detected on intact cells and on membranes washed either by filtration or sedimentation.

Gel Electrophoresis and Autoradiography

The formation of the direct linkage complex was examined using the discontinuous pH system of Laemmli [25]. Best results were obtained with slab gels having a 5–12% gradient acrylamide resolving gel, measuring 1.5 mm thick × 150 mm wide × 180 mm long, topped with a 20 mm high stacking gel. Intact cells and membranes were solubilized in ESB, and boiled for 5 min prior to their application to a gel. When intact cells were used, the monolayers were solubilized by the addition of 0.1 ml of ESB per 10 cm^2 of growth area, and the resulting highly viscous solution was subjected to vigorous shearing through a 20–22-gauge hypodermic needle prior to boiling. Gels were stained with Coomassie blue, destained, dried on filter paper, and autoradiographed on Kodak x-ray film. Only the resolving gels, which contained the bulk of the applied radioactivity in all cases, have been photographically reproduced in this paper. Relative molecular weights were determined by comparison with the mobilities of red blood cell ghost proteins, which were visualized by staining, and by using the published MW values for bands 1, 2, 3, 4.1, 4.2, and 5 [26]. BSA was present in all binding assays and was visible as a contaminant in most samples as a consequence of its binding to membranes; it served as a convenient marker of MW = 68,000. A plot of log MW versus mobility was linear in the MW = 70,000–240,000 region. Identical results were obtained with a commercially available (Bio Rad) kit with molecular weight standards ranging from 43,000 to 200,000. The molecular weights reported here are strictly relative, and as such, differ slightly from those previously reported for EGF–EGF receptor direct linkage complexes [15, 27]. The slight deviation from agreement with previous results is reasonable considering the differences in gel systems and molecular weight standards used.

Determination and Quantitation of Radioactivity in the Directly Linked Complex

The regions of the dried gel which contained direct linkage complexes were localized by autoradiography and excised, and the radioactivity was measured by gamma scintillation spectrometry. The radioactivity present in direct linkage complexes was corrected for background radioactivity in adjacent regions of the same lane or corresponding regions of a lane containing an identical sample, where excess unlabeled EGF was included during the initial incubation.

Chemical Modification of EGF

Synthesis of biotinyl EGF. N-hydroysuccinimidyl biotin (NHS Biotin) was synthesized by dicyclohexyl carbodiimide-mediated condensation of biotin and N-hydroxysuccinimide [28]. Acylation of EGF was accomplished at alkaline pH, a condition that predominantly modifies free amino groups [29], of which EGF has only one, the amino terminus [30]. One hundred micrograms (16.7 nmoles) of EGF in 0.147 ml of H_2O were mixed with 0.153 ml of 0.5 M Na_2CO_3 at pH 9.0, containing 0.3% NaN_3 and 0.1% Triton X-100. Modification was accomplished by the addition of 8 sequential aliquots of NHS biotin (0.286 ng or 0.84 μmoles in 5 μl of dry dimethyl formamide) during a 2 h incubation at 20°C. The reaction was terminated by the addition of 20 μmoles of ammonium acetate in 10 μl of H_2O, and the solution was stored at –20°C. The resulting biotinyl EGF was iodinated using chloromine T to specific activities comparable to those achieved with native EGF. Chromatography on Sephadex G-10, which was routinely used to separate ^{125}I-EGF from free ^{125}I, also served to purify ^{125}I-biotinyl EGF (as well as iodinated forms of the other derivatives) to an extent that permitted their use in the described experiments. The extent of acylation of EGF was estimated by mixing ^{125}I-biotinyl EGF with an excess of avidin and subjecting the mixture to column chromatography on Sephadex G-50. ^{125}I-biotinyl EGF was included in the column, but when avidin was added, more than 80% of the applied radioactivity was eluted in the void volume. When avidin was mixed with excess free biotin and then with ^{125}I-biotinyl EGF, the radioactivity was eluted from Sephadex G-50 in the position of free ^{125}I-biotinyl EGF. We conclude that the preparation of biotinyl EGF used in these experiments was at least 80% modified with NHS biotin, presumably at the amino terminus. In order to determine the amount of unmodified EGF present in this preparation, we subjected biotinyl EGF to DEAE chromatography at pH 7.1 (Fig. A-3, Appendix). This procedure separates native EGF from amino modified EGF [10]. Two EGF-containing peaks were detected. One, which represented less than 5% of the total EGF in the preparation, had a reduced ability to bind avidin relative to the material in the major peak and probably contained unmodified EGF. Because of its high avidin binding capacity, the major peak contained biotinyl EGF. Fractions taken from across this peak displayed a constant amount of non-avidin-binding material (approximately 20%). Since the non-avidin-binding material in the major peak is not readily separable from that which does bind avidin, it most likely does not represent unmodified EGF. The existence of non-avidin-binding material may result from the modification of EGF with a form of biotin incapable of binding to avidin. This derivative could have been present originally as an impurity in the commercial biotin preparations, or it could have been formed by chloramine T oxidation during the iodination procedure. The amount of unmodified EGF in this biotinyl EGF preparation (less than 5%) was not sufficient to explain the residual direct linkage complex forming ability of biotinyl EGF (see Table IV).

Trypsin treatment of biotinyl EGF. Biotinyl EGF was subjected to limited trypsin treatment to prepare an EGF derivitive lacking the carboxy terminal 5 amino acids [30]. Twenty five micrograms of EGF (4.2 nmoles) were treated with TPCK-treated trypsin at an EGF:trypsin ratio of 300:1 (w:w). The mixture was incubated for 2 h at 37°C. The product was stored without purification at –20°C.

Iodoacetate modification of EGF. EGF was treated with iodoacetate under conditions reported to lead to modification of histidine and, to a lesser extent, methionine residues [31]. Twenty-five micrograms of EGF (4.2 nmoles EGF, containing 4.2 nmoles

histidine) in 37 μl of H_2O were mixed with 63 μl of 0.2 M sodium acetate at pH 5.6, containing 3.2 M urea. An excess of iodoacetic acid (107 nmoles in 10 μl of H_2O) was added, and the mixture was incubated under an atmosphere of toluene at 30°C for 48 h. The product was stored at −20°C.

Reduction and carboxymethylation of EGF. EGF was reduced essentially by the procedure of Savage et al [30]. Twenty-five micrograms of EGF (4.2 nmoles) in 37 μl of H_2O were mixed with 63 μl of 0.1 M sodium phosphate at pH 8.5, containing 8 M urea, and 2-mercaptoethanol (1μl) was added. The mixture was maintained under an atmosphere of toluene at 30°C for 48 h. The reduced EGF was alkylated with iodoacetic acid (15 μmoles added in 10 μl of 0.1 M sodium phosphate, at pH 8.5) for 5 h in the dark and stored at −20°C. The specific activity of this derivitive after chloromine T-mediated iodination was considerably less than that of iodinated native EGF.

Determination of EGF-Stimulated ^{3}H-Thymidine Incorporation Into DNA

3T3 cells were plated in DMEM plus 10% fetal calf serum. After 48 h this medium was replaced with starvation medium (DMEM plus 0.8% fetal calf serum); and the cells were starved for 3 days. The efficacy of this procedure in producing quiescent cultures was experimentally confirmed by in situ autoradiography. To initiate the G–S transition, the medium was replaced with DMEM + 0.8% fetal calf serum containing EGF. At the time of maximal DNA synthesis during S phase, a time determined in preliminary experiments to by 18 h, ^{3}H-thymidine was added for 2 h at 37°C, and the cells were processed for determination of ^{3}H-thymidine uptake into DNA [13].

Determination of Protein Concentration

Protein was determined by either a conventional [32] or modified [33] Lowry procedure, using BSA as the standard. In one instance (see Table II) the total cellular protein of the intact A431 cells used to determine ^{125}I-EGF binding was measured using a dye binding assay [34], again using BSA as the standard.

RESULTS

Selection of a Source of Material for Characterization of the Direct Linkage Complex

Direct linkage complex formation was observed initially on intact 3T3 cells [15]. A small portion of the ^{125}I-EGF that was bound specifically to these cells migrated as an MW = 160,000 protein having the characteristics of an EGF–EGF receptor complex (Fig. 1, lane A). A small amount of an additional complex of MW = 145,000 was sometimes observed; the formation of both was inhibited when binding proceeded in the presence of unlabeled EGF (lane B). Direct linkage complexes of the same MW are formed with other cell lines (data not shown). Rapid lysosomal processing of the complexes [15] limits the usefulness of intact cells for studying the direct linkage process. We therefore used isolated membranes, which lack the normal cellular degradative pathway.

Membranes from a variety of sources were surveyed for direct linkage complex forming ability. Membranes from 3T3 cells, HF-15 cells (normal human foreskin fibroblasts), and A431 cells (a human tumor line that hyperproduces EGF receptors [4]) give rise to several direct linkage complexes; the formation of all is inhibited by excess unlabeled EGF (Fig. 1, lanes C–H). The lower molecular weight direct linkage complexes arise as the result of endogenous proteolytic modification of EGF receptors during membrane preparation [18]; these modifications do not detectably alter the affinity of EGF receptors for

TABLE I. Direct Linkage Complex Formation in Membrane Preparations From Different Cell Types*

	Source of membranes	Specific activity ^{125}I-EGF binding (pmoles/mg)	Bound EGF in direct linkage complexes (%)
Experiment 1[a]	3T3	0.50	1.45
	HF-15	0.59	0.72
	A431	8.0	0.78
Experiment 2[b]	Human placenta	0.53	0.78

*Experimental details are described in Figure 1 and Experimental Procedures. Crude membranes were prepared from cultured cells by Procedure I (Experimental Procedures). Human placental membranes were prepared according to O'Keefe et al [6]. After electrophoresis, the radioactivity present in all forms of the direct linkage complex was measured, corrected for background radioactivity and expressed as a percentage of the total radioactivity applied to the gel.
[a]Assays contained 8.3 nM ^{125}I-EGF (2.6 × 10^9 cpm/nmole) and 64 μg (3T3), 54 μg (HF-15), or 80 μg (A431) of membrane protein. The incubation was for 1 h at 20°C.
[b]The assay contained 8.3 nM ^{125}I-EGF (1.6 × 10^9 cpm/nmole) and 160 μg of human placental membrane protein. The incubation was for 4 h at 20°C.

^{125}I-EGF. The percentage of specifically bound ^{125}I-EGF in all direct linkage complex components varied in different experiments and was usually less than 2% of the total radioactivity bound to membranes from all sources (Table I). Although the ratio of direct linkage complex formed to EGF bound was similar for all membranes studied, those derived from A431 cells bound extraordinarily high amounts of EGF and gave rise to correspondingly high amounts of direct linkage complexes. Therefore A431 membrane preparations were used in most experiments.

Effects of Time and Temperature on Direct Linkage Complex Formation in A431 Membranes

Binding of ^{125}I-EGF to its receptor on A431 membranes reaches a maximum within 15 min at temperatures ranging from 0° to 37°C, but direct linkage occurred more slowly (Fig. 2) [15]. The rate of direct linkage complex formation was decreased more by low temperature than was binding (data not shown and [15]). The rate of formation of all major direct linkage complex forms is similar (Fig. A-1, Appendix), showing that the lower molecular weight components do not arise by proteolytic cleavage of 160,000 dalton EGF-linked progenitor.

Dependence of Direct Linkage on EGF Concentration

The susceptibilities of ^{125}I-EGF binding and direct linkage complex formation to competition by unlabeled EGF were similar (Fig, 3; Fig. A-2, Appendix). When ^{125}I-EGF was present at half-saturating concentration, half-maximal inhibition of both binding and direct linkage complex formation was achieved at an unlabeled EGF concentration of 87 nM. Saturation of direct linkage complex forming activity with ^{125}I-EGF was similar to that of the binding reaction (data not shown) [15]. The percentage of bound EGF involved in complex formation with A431 receptors varied somewhat with increasing EGF concentration, ranging from 0.33% at 14 nM, to 0.21% at 112 nM. The K_F of direct linkage com-

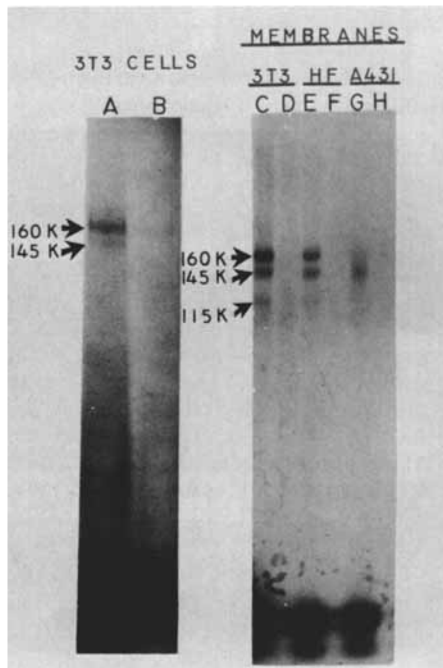

Fig. 1. Direct linkage complex formation in intact 3T3 cells and isolated membranes from different cell types. Cells: confluent 3T3 cell monolayers were incubated with 16.7 nM ^{125}I-EGF (1.2×10^9 cpm/nmole) in DBH. One set of cultures contained unlabeled EGF at 158 nM. After the standard binding reaction (Experimental Procedures), the cells were washed and solubilized in ESB, and an aliquot of 200 μl was subjected to electrophoresis. Membranes: Membranes from 3T3, HF-15, and A431 cells were isolated by Procedure I (Experimental Procedures). The binding assays contained 8.3 nM ^{125}I-EGF (2.6×10^9 cpm/nmole); 2.4 μM unlabeled EGF (where indicated) and membrane protein at 400 μg/ml (3T3); 340 μg/ml (HF) or 500 μg/ml (A431) in DBH. Binding was arrested by sedimentation through a sucrose shelf, the membrane pellet was washed with DMEM and then solubilized with ESB, and the solubilized proteins were subjected to electrophoresis (Experimental Procedures). Arrows indicate the molecular weights of the direct linkage products relative to the mobilities of the standards visible on the stained, dried gel. The formation and significance of each product is discussed in the text. Lanes: A, direct linkage complexes formed with intact 3T3 cells; B, as in A, plus unlabeled EGF; C, direct linkage complexes formed with 3T3 membranes; D, as in C, plus unlabeled EGF; E, direct linkage complexes formed with HF-15 membranes; F, as in E, plus unlabeled EGF; G, direct linkage complexes formed with A431 membranes; H, as in G, plus unlabeled EGF.

plex formation (a constant analagous to the K_A of ^{125}I-EGF binding) can be utilized to describe the concentration dependence of direct linkage. In A431 membranes, the K_F values for all forms of the direct linkage complex are similar, although not identical, to the K_A of ^{125}I-EGF binding (data not shown). A similar correspondence of K_F and K_A was observed with 3T3 cells, which bind EGF with an affinity at least 10 times greater than A431 [15]. The data are consistent with the conclusion that binding of ^{125}I-EGF to its receptor is obligatory for direct linkage complex formation.

Chemical Stability of the Direct Linkage Complexes

Direct linkage complex formation was influenced by pH. The percentage of bound radioactivity present in direct linkage complexes was constant at pH values less than neutrality but was increased at higher pH values. Once formed, direct linkage complexes

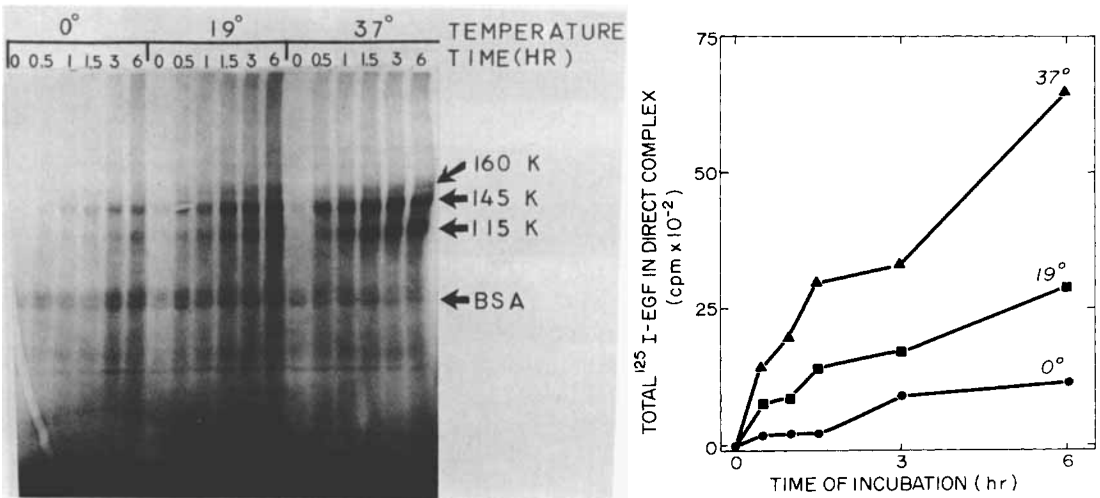

Fig. 2. Time and temperature dependance of direct linkage in A431 membrane. Samples containing 190 μg of A431 membrane protein in 0.59 ml of DBH (plus 0.01% BSA) were equilibrated at the indicated temperatures for 5 min. Binding was initiated by the addition of 0.11 ml of ^{125}I-EGF (1.7×10^9 cpm/nmole) to give a final concentration of 88 nM. At the indicated times 0.1 ml aliquots were removed, and the membranes were collected and washed by sedimentation, frozen, and solubilized in ESB immediately prior to electrophoresis. Left panel: autoradiogram showing the time and temperature dependence of direct linkage complex formation. Arrows indicate the relative molecular weights of each direct linkage component. The position of the complex migrating with BSA is also indicated. Right panel: quantitation of radioactivity in direct linkage complexes as a function of time and temperature. The radioactivity in all direct linkage complexes was determined and corrected for the amount of radioactivity present at time zero. The time and temperature dependences of the formation of each individual component are presented in Figure A-2, Appendix.

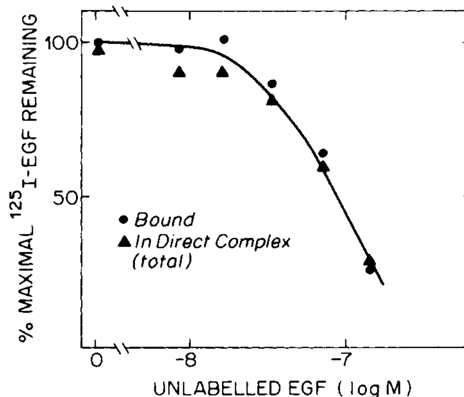

Fig. 3. Competition of unlabeled EGF for ^{125}I-EGF binding and direct linkage complex formation with A431 membranes. Experimental conditions were identical to those given in Figure 5, except that the ^{125}I-EGF concentration was held constant at 28 nM and the concentration of unlabeled EGF was varied from 0 to 143 nM. The K_d of this membrane preparation for ^{125}I-EGF was 29 nM. ^{125}I-EGF was thus present at a concentration sufficient for half-maximal receptor occupancy. •—• ^{125}I-EGF binding in the presence of increasing amounts of unlabeled EGF. Each point is the average of duplicate determinations. Maximal binding was 0.14 pmoles, which was approximately 90% specific. ▲—▲ Total radioactivity in direct linkage complexes. Maximal radioactivity present in the major forms of the direct linkage complex was 0.78 fmoles. The competition for the formation by each individual direct linkage complex was virtually identical and is presented in Figure A-1 (Appendix).

were stable in solution at pH values ranging from 3.5 to 11.5 (Table II). Direct linkage complexes were also insensitive to hydroxylamine, another condition that disrupts ester linkages [31, 35]. The direct linkage complexes are thus markedly stable and probably are covalent products.

Effects of Chemical Modification of EGF on Direct Linkage

EGF was subjected to various chemical modifications, and the derivitives were assayed for both EGF binding and direct linkage complex formation (Table III). Of the various derivatives tested, only biotinyl EGF bound to 3T3 cells equally as well as native EGF and had a reduced ability to engage in direct linkage complex formation. Removal of the 5-COOH terminal amino acid residues of biotinyl EGF by limited trypsin treatment [30] reduced its binding ability but did not further lower the extent to which the bound derivitive formed direct linkage complexes. A more detailed experiment confirmed the initial observation that biotinyl EGF was reduced in its ability to form direct linkage complexes. Native and biotinyl EGF exhibited almost identical K_d values for binding to 3T3 cells (Fig. 4), but the number of direct linkage complexes formed by biotinyl EGF in the same experiment was reduced by 63 ± 7% (Table IV). The reduction in direct linkage was less than the extent of modification of the EGF in this preparation by biotin (at least 80%, as discussed in Experimental Procedures). This disparity suggests that the observed inhibition is not due simply to blocking of a nucleophilic site – presumably the amino terminus of EGF – involved in covalent bond formation. Instead, acylation may result in the formation of a conformationally altered EGF molecule, in which the site(s) involved in complex formation is sterically shifted to a position unfavorable for direct linkage complex formation. Alternatively, the amino terminus may be one, but not the only, site on the EGF molecule involved in covalent bond formation giving rise to direct linkage complexes. The observed inhibition may reflect only the extent of the amino terminus participation in direct linkage.

TABLE II. Chemical Stability of Direct Linkage Complexes*

Treatment	Radioactivity in direct linkage complexes (%)
None	2.1
0.1 N acetic acid	1.9
0.1 N NaOH	2.0
0.45 M hydroxylamine	1.8

*Membrane protein (36 μg) was mixed with 67 nM ^{125}I-EGF (1.1×10^9 cpm/nmole) in a volume of 1 ml of 10 mM sodium phosphate at pH 8.5 containing 0.3 M sucrose, and the samples were incubated for 1.5 h at 37°C. The membranes were collected by sedimentation and suspended in 1 ml of the same solution at pH 7.4. Duplicate 90 μl aliquots of this suspension were mixed with 10 μl of H_2O (as a control), 1 N acetic acid (to give a final pH of 3.5), 1 N NaOH (final pH was 11.5), or 4.5 M hydroxylamine at pH 7.0. After a 5 h incubation at 20°C, all samples were neutralized, mixed with twice-concentrated ESB, and subjected to electrophoresis. The amount of radioactivity present in direct linkage complexes was determined and is expressed as percentage of the total radioactivity applied to the gel.

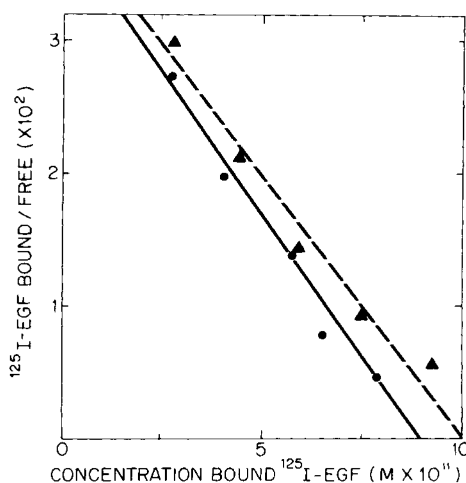

Fig. 4. Binding of native and biotinyl EGF. Duplicate cultures of 3T3 cells grown on 28 cm^2 plastic dishes were treated with ^{125}I-native or biotinyl EGF at 1.0, 2.1, 4.2, 8.3, and 16.7 nM, and nonspecific binding was determined in samples also incubated with 118 nM unlabeled EGF. The specific activity of native EGF was 1.68×10^9 cpm/nmole; biotinyl EGF, 1.43×10^9 cpm/nmole. After thorough washing, monolayers were solubilized in ESB, and cell-bound radioactivity was determined. Specific binding is plotted according to Scatchard [24]. ▲- -▲ Specific binding of ^{125}I-native EGF. ●—● Specific binding of ^{125}I-biotinyl EGF.

TABLE III. Effects of Chemical Modification of EGF on EGF Binding and Direct Linkage Complex Formation*

	Specific binding (% of untreated EGF)	EGF in direct linkage complexes (% of specific binding)
None	100	1.33
Iodoacetate	127	1.08
N-hydroxysuccinimidyl biotin	109	0.53
N-hydroxysuccinimidyl biotin, followed by trypsin treatment	48	0.60
2-Mercaptoethanol	0	0

*EGF was subjected to chemical modification with several site-specific reagents (Experimental Procedures). The derivitives were radioiodinated by the chloramine T procedure to the following specific activities: iodoacetate-treated EGF, 2.4×10^9 cpm/nmole; 2-mercaptoethanol-treated EGF, 0.23×10^9 cpm/nmole; biotinyl EGF, 2.4×10^9 cpm/nmole; trypsin-treated, biotinyl EGF, 3.7×10^9 cpm/nmole. Native EGF was radioiodinated to a specific activity of 3.2×10^9 cpm/nmole. After radioiodination, each derivitive was incubated at 5.3 nM in DBH with 3T3 cells for 1 h at 37°C. The binding of each derivitive was measured on duplicate monolayers and corrected for the nonspecific binding measured in the presence of 167 nM unlabeled EGF. Binding is expressed as a percentage of the amount of iodinated native EGF bound per monolayer (27 fmoles). The amount of bound EGF present in the direct linkage complexes was then determined as described in Experimental Procedures.

Biological Properties of Native and Biotinyl EGF

The reduced ability of biotinyl EGF to form direct linkage complexes presented an opportunity to test the role of direct linkage in EGF-induced DNA synthesis. The same preparations of native and biotinyl EGF assayed for their abilities to form direct linkage complexes (Table IV) were compared for their abilities to stimulate the uptake of ^{3}H-thymidine into DNA (Fig. 5). In two separate experiments, the concentration dependence and both the half-maximal (Fig. 5, right) and maximal extent of stimulation (Fig. 5, left) were identical for native and biotinyl EGF. Since direct linkage was inhibited by over 60% without affecting the dose-response curve for EGF-induced stimulation of ^{3}H-thymidine into DNA, it is unlikely that it serves in a role causal to the biological activity of EGF. In another experiment, native and biotinyl EGF were tested for their abilities to stimulate EGF receptor down regulation at low physiological EGF concentrations. No differences were observed, indicating no involvement of direct linkage in triggering of EGF receptor down regulation (data not shown).

Does a Restricted Subclass of EGF Receptors Engage in Direct Linkage Complex Formation?

The low yield of direct linkage complex formation relative to EGF binding raised concern that a small subclass of EGF receptors, possibly one not representative of the total, participated in complex formation. This could severely limit the usefulness of direct linkage complex formation as an affinity labeling technique. The test for this possibility is described in Table V. If membranes are first incubated with unlabeled EGF or EGF derivatized with nonradioactive iodine, a small reactive subclass of EGF receptors might engage

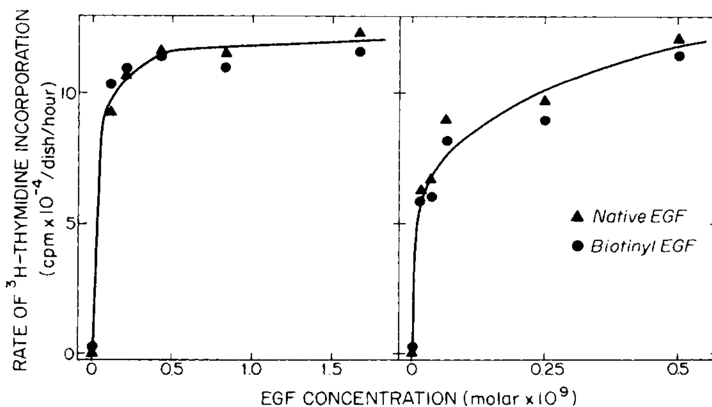

Fig. 5. Biological activity of native and biotinyl EGF. Quiescent cultures of 3T3 cells (prepared on 2 cm^2 plastic wells as described in Experimental Procedures) were incubated with 1 ml of DMEM plus 0.8% fetal calf serum containing native or biotinyl EGF at the indicated concentrations. After 18 h at 37°C, 10 μl of ^{3}H-thymidine (50 Ci/mmole, diluted to 0.1 mCi/ml with DMEM), was added to each culture. After an additional 2 h at 37°C, acid-precipitable radioactivity was measured by liquid scintillation counting as described in Experimental Procedures. Each point is the average of duplicate determinations corrected for the radioactivity incorporated in the absence of EGF (36,200 cpm/dish/h for the left panel and 108,300 cpm/dish/h for the right panel. In the experiment in the left panel, each well contained an average of 17 μg of total protein; protein in the experiment in the right panel was not determined. Left panel: rate of thymidine incorporation by native (▲) and biotinyl EGF (●) over a wide range of EGF concentrations. Right panel: rate of thymidine incorporation at low EGF concentrations.

in the formation of unlabeled direct linkage complexes, thereby reducing the amounts of labeled direct linkage complexes formed during a second incubation with ^{125}I-EGF. Alternatively, if the EGF receptors that engage in direct linkage complex formation are members of a larger class that is more representative of the total population, then the small amount of unlabeled complex formed after even an extended incubation period would not noticeably alter the number of direct linkage complexes formed later with ^{125}I-EGF. The data presented in Table V support the second alternative. Prior incubation of membranes with noniodinated EGF or with ^{127}I-EGF was ineffective in reducing subsequent direct linkage complex formation with ^{125}I-EGF.

TABLE IV. Direct Linkage Complex Formation by Native and Biotinyl EGF*

	^{125}I-EGF in direct linkage complexes (moles × 10^{16})		
^{125}I-EGF (nM)	Native	Biotinyl	% Reduction
1.04	4.88	1.78	63.5
2.08	12.3	3.56	71.1
4.17	12.5	5.88	53.0
8.3	12.1	4.87	59.8
16.7	17.9	5.77	67.8
		Mean	63.0
		SD	7.06

*Aliquots (100 μl) of each of the samples used to determine specific binding in Figure 10 were subjected to electrophoresis for the determination of the amount of bound EGF in direct linkage complexes.

TABLE V. Effects of Prior Incubation of A431 Membranes With Unlabeled EGF or ^{127}I-EGF on Direct Linkage Complex Formation*

	Second incubation (24–48 h)	
Addition, first incubation (0–24 h)	^{125}I-EGF bound (fmoles)	^{125}I-EGF in Direct linkage complexes (fmoles)
None	161	3.8
Unlabeled EGF	151	3.1
^{127}I-EGF	182	3.3

*Duplicate aliquots of membrane protein (72 μg, Procedure III) in PBS plus 0.5% NaN_3 were mixed with no EGF, 67 nM unlabeled EGF, or 67 nM ^{127}I-EGF (prepared as described [12], but using non-radioactive Na ^{127}I instead of Na^{125}I), and incubated for the first 24 h period at 37°C. Membranes were collected by sedimentation, washed and suspended in PBS plus 0.5% NaN_3 containing 67 nM ^{125}I-EGF (3.2 × 10^9c pm/nmole). Samples were then incubated for a second 24 h period at 37°C, washed again by sedimentation, and dissolved in ESB. Membrane-bound radioactivity was determined and corrected for the radioactivity bound in the presence of 5 μM unlabeled EGF (25% of the total). After electrophoresis, the radioactivity present as direct linkage complexes was determined as described in Experimental Procedures. During the first 24 h incubation, identical prepared samples specifically bound 223 fmoles of ^{125}I-EGF, of which 6.57 fmoles was in direct linkage complexes.

DISCUSSION

We have extended our previous observation that specific binding of radioiodinated EGF is accompanied by direct linkage of EGF to its receptors on intact cells and isolated membranes from a variety of sources [15]. Evidence that direct linkage complexes represent ^{125}I-EGF attached to its receptors can be summarized as follows: 1) The direct linkage complexes and the EGF–EGF receptor complex produced by photoaffinity labeling have identical electrophoretic behavior [12, 15]. 2) The complexes have biological and chemical properties expected of EGF–EGF receptor complexes [15] – eg, production of the same lysosomal processing products noted previously with photoaffinity labeled EGF receptors [13]. 3) Cell surface proteins having properties identical to those of the direct linkage complexes can be identified by surface-specific iodination of EGF-responsive cells [36]. 4) After A431 membranes have been solubilized with solutions containing Triton X-100, direct linkage complex forming activity cofractionates with ^{125}I-EGF binding activity during gel chromotography and velocity sedimentation [42].

The formation of direct linkage complexes in A431 membranes requires EGF binding; its dependence on ^{125}I-EGF concentration and susceptibility to inhibition by unlabeled EGF are similar to those of the binding reaction. Complex formation is markedly slower than binding. The direct linkage complex formed with intact 3T3 cells is precipitated from detergent solution by an antiserum to EGF [15]. These observations indicate that direct linkage involves attachment of a relatively intact EGF molecule to its receptor(s) during a reaction that occurs after binding.

The direct linkage complexes once formed are stable, and no test performed to date provides insight into the mechanism of formation. The complexes are not disrupted by boiling in SDS solutions containing 2-mercaptoethanol. Nor are they disrupted by extremes of pH or treatment with high concentrations of a strong nucleophile, hydroxylamine. Direct linkage complex formation is not affected by inhibitors of transglutaminase reactions [15] – eg, EDTA or exogenously added amines [37]. Specific interference with direct linkage was achieved by acylation of EGF with a derivative of biotin. Since the extent of inhibition of direct linkage (63%) is substantially less than the extent of EGF acylation (at least 80%), the observed inhibition may not be due to the modification of a specific amino acid residue involved in covalent bond formation. Since EGF and biotinyl EGF have identical dose-response curves for stimulation of DNA synthesis in 3T3 cells, direct linkage complex formation is not essential for this cellular response to EGF.

We have not completely excluded the possibility that direct linkage results from a chemically reactive EGF derivitive formed during the harsh oxidative conditions necessary for iodination. This possibility is not supported by the following evidence. First, ^{125}I-EGF forms the direct linkage complexes after several weeks of storage in aqueous solution. Second, direct linkage complexes were formed to a similar extent when the ^{125}I-EGF was prepared by a milder procedure than that used here [36]. Third, prior incubation of membranes with ^{127}I-EGF does not inhibit subsequent direct linkage complex formation with ^{125}I-EGF to a greater extent than does unlabeled EGF (Table V). Fourth, the ability of ^{125}I-EGF to form direct linkage complexes was not decreased by its prior incubation with membranes for periods of up to 24 h at 37°C (data not shown). We have made several attempts to use antiserum to EGF to precipitate direct linkage complexes from nonionic detergent extracts of metobolically labeled cells incubated with unlabeled EGF. In preliminary studies [15], a protein similar in size to the direct linkage complex was immune-

precipitated. We subsequently became aware that EGF binding to the EGF receptor persists in detergent solution [42], even though at that time there were reports in the literature that EGF binding activity was inactivated by detergent treatment [14, 38].

There is precedent in the literature for the formation of a covalent complex between two reversibly bound proteins. Chymotrypsin forms significant amounts of covalent intermediates with its substrates, including the insulin B chain, during the initial burst of its catalytic activity [39]. Kunitz soybean trypsin inhibitor and trypsin also form a covalent adduct [40]. The latter finding is particularly noteworthy, as EGF has been shown to possess a distinct structural homology with another trypsin inhibitor known as pancreatic secretory trypsin inhibitor [41]. These complexes are not identical with the direct linkage complexes. They can be isolated only under extremely acidic conditions, and once isolated they are disrupted under alkaline conditions, to which the direct linkage complexes are impervious (Table IV) [40]. The low yield of direct linkage complex formation could indicate a vestigial function of the EGF molecule, or at least one unrelated to its mitogenic activity. Interestingly, the covalent complex formed between thrombin and its receptor on human fibroblasts [16] closely resembles a reported linkage between thrombin and antithrombin III, a prominent inhibitor of thrombin in serum [43].

Regardless of the mechanism of direct linkage complex formation or its biological significance, this process remains an exquisitely simple and powerful technique for EGF receptor affinity labeling. This has enabled us to make several observations concerning the transmembrane distribution of functional sites on EGF receptors [18].

ACKNOWLEDGMENTS

This work was supported by grant BC-79 from the American Cancer Society and in part by USPHS grant AM 25826-01 during the terminal stages. P.L. was recipient of a predoctoral USPHS-National Research Service Award in Tumor Cell Biology (CA 09056) and also received support from an American Cancer Society Institutional grant (IW-131) to the UCLA Jonsson Comprehensive Cancer Center.

We wish to thank Cindy Blifeld for invaluable assistance at the initial stage of this work, Pamela Billings for assistance in the development of a membrane preparative procedure, and Betty Handy for typing the manuscript.

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APPENDIX

TABLE A-I. Distribution of Membrane Markers Upon Subcellular Fractionation*

Fraction	Protein (mg)	^{125}I-EGF binding activity: Total (pmoles)	^{125}I-EGF binding activity: Specific activity (pmoles/mg)	N-Acetyl-β-D-glucosaminidase activity: Total (A_{400}/h)	N-Acetyl-β-D-glucosaminidase activity: Specific activity (A_{400}/h/mg)
Experiment I					
Whole cell	825	7331 (100)[a]	8.89	–	–
2,600g supernatant	224	3573 (48.7)	16.0	–	–
35,000g pellet	–	2321 (31.7)	–	–	–
Band					
0/12	7.2	513 (7.0)	71.2	–	–
12/25	6.0	267 (3.6)	44.5	–	–
25/35	3.2	107 (1.5)	33.4	–	–
Experiment II					
Whole cell	1040	9460	9.10	1040	1.0
Band					
0/12	3.6	297	82.5	1.14	0.32
12/25	3.2	184	57.5	2.08	0.65
25/35	3.2	143	44.7	1.28	0.40

*Membranes were prepared by Procedure II (Experimental Procedures) from A431 cells grown in roller culture on approximately 10,000 cm^2 surface area. The indicated fractions were assayed for membrane marker activities in order to assess purification. EGF binding was measured at an ^{125}I-EGF concentration of 25 nM (1.7×10^9 cpm/nmole). The lysosomal marker, N-acetyl-β-D-glucosaminidase, was assayed by a modification of a published procedure [21], using p-nitrophenyl N-acetyl-β-D-glucosaminide as a substrate. Triton X-100 was added to a final concentration of 0.5% (v:v) prior to assay to improve substrate accessibility. Color was developed by the addition of 1 ml of a 0.1 M Na_2CO_3 solution at pH 10.7, containing 0.1% SDS, and the increase in absorbance at 400 nm was recorded.

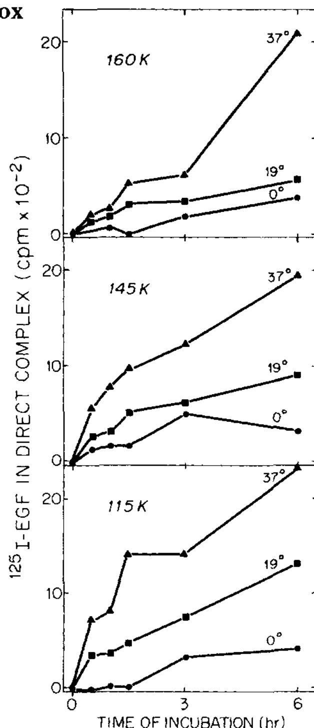

Fig. A-1. Time and temperature dependence of the formation of the individual direct linkage complexes. The time and temperature dependence of the formation of each individual direct linkage complex was determined in the experiment presented in Figure 2 of the text. Approximately 21% of the radioactivity in direct linkage complexes was present as the MW = 160,000 component, 36% in the MW = 145,000 component, and 43% in a MW = 115,000 component. (●—●) Direct linkage complexes were formed at 0°; (■—■), 19°; (▲—▲), or 37°. Top panel: Formation of the MW = 160,000 component of the direct linkage complex. Middle panel: Formation of the MW = 145,000 component of the direct linkage complex. Bottom panel: Formation of the MW = 115,000 component of the direct linkage complex.

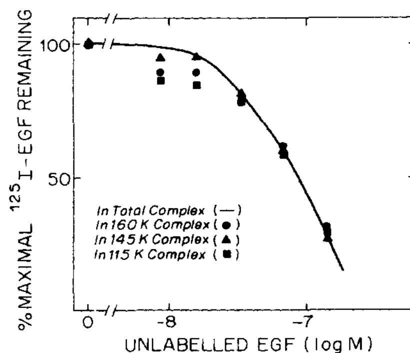

Fig. A-2. Competition by unlabeled EGF for the formation of each individual direct linkage complex. The ability of unlabeled EGF to compete for the direct linkage of ^{125}I-EGF to each individual proteolytic product of the EGF receptor was determined for the experiment presented in Figure 3 of the text. ^{125}I-EGF was present at a half-saturating concentration. Of the total radioactivity present as direct linkage complexes, 25% was in the form of a MW = 160,000 component, 35% was present as a MW = 145,000 component, and 39% was present as a MW = 115,000 component. —— Competition for total ^{125}I-EGF in all forms of the direct linkage complex (0.785 fmoles total). ● ● Competition for ^{125}I-EGF in the MW = 160,000 direct linkage complex. ▲ ▲ Competition for ^{125}I-EGF in the MW = 145,000 direct linkage complex. ■ ■ Competition for ^{125}I-EGF in the MW = 115,000 direct linkage complex.

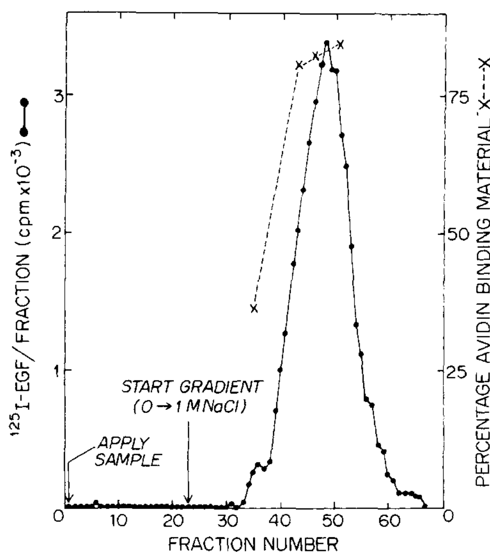

Fig. A-3. Fractionation of biotinyl EGF by analytical ion-exchange chromatography. Unlabeled EGF (85 nmoles in 1.2 ml of an aqueous 0.5 M Na_2CO_3 at pH 9.0, containing 0.1% Triton X-100 and 0.3% NaN_3) was mixed with a trace quanity of ^{125}I-EGF to achieve a final specific activity of 9.0×10^5 CPM/μmole. EGF was modified with NHS biotin as described in Experimental Procedures. Unreacted NHS biotin was first removed by chromatography on Sephadex G-25 equilibrated with 0.02 M Tris•HCl at pH 7.1, and the EGF was then applied to DEAE cellulose for separation of unreacted EGF from acylated EGF [10]. One milliliter fractions were collected, and EGF-containing fractions were detected by monitoring the effluent for radioactivity. Total recovery of EGF from the column was determined to be 86%. Individually iodinated aliquots of the indicated fractions were assayed for avidin binding as described in Experimental Procedures. Only a small amount of material was eluted in the position characteristic of native EGF (fractions 33–40).