Journal of Supramolecular Structure 14:511–525 (1980)
Membrane Transport and Neuroreceptors 327–341

Properties of Receptors for Epidermal Growth Factor in Detergent Solution: Evidence for Heterogeneous Aggregated States

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

Between 60% and 100% of epidermal growth factor (EGF) binding activity was recovered from membranes of the A431 human epidermoid carcinoma cell line treated with solutions containing the nonionic detergent Triton X-100. Approximately half of the recovered binding activity was sedimented at low centrifugal force and hence was operationally insoluble in nonionic detergent solution. Receptors in both the detergent-soluble and -insoluble fractions displayed similar affinities for ^{125}I-EGF, and the values were in good agreement with those obtained for receptors in untreated membranes. The receptors in both fractions also formed identical direct linkage complexes with ^{125}I-EGF in similar yield, providing no evidence for partitioning of different molecular species of EGF receptors in the detergent-soluble and -insoluble fractions.

Gel chromatography of the detergent-soluble membrane fraction on Sepharose 6-B revealed heterogeneity of ^{125}I-EGF binding activity; the smallest and most monodisperse peak of activity resolved by this technique was eluted at a Stokes radius of 95 Å. Operationally soluble ^{125}I-EGF binding activity also behaved heterogeneously during velocity sedimentation; more than half the activity sedimented more rapidly than the apparently monidisperse, 7S form. An average of less than half the nonionic detergent-solubilized activity recovered from 10 independent membrane preparations behaved as an apparently monodisperse entity. Since a maximum of 60% of ^{125}I-EGF binding activity was operationally soluble, less than 25% of the total EGF binding activity was recovered in an apparently monodisperse form. The remaining 75% of the EGF receptors displayed a marked tendency to exist as aggregates in nonionic detergent solutions.

Key words: EGF receptors – solubilization, aggregates in nonionic detergent, gel filtration chromatography, zonal sedimentation, on A431 membranes

Abbreviations: EGF, epidermal growth factor; SP buffer, 0.3 M sucrose in 0.1 M sodium phosphate at pH 7.4; ST buffer, 0.25 M sucrose in 10 mM Tris·HCl at pH 7.4; DMEM, Dulbecco's modified Eagle medium; DBH, DMEM minus bicarbonate plus 0.1% BSA in 10 mM HEPES, pH 7.4; PEG, polyethylene glycol 6000; SDS, sodium dodecyl sulfate; ESB (electrophoresis sample buffer), 63 mM Tris·HCl at pH 6.8, 3% SDS and 10% glycerol; PBS, 0.15 M NaCl in 0.1 M sodium phosphate at pH 7.4; MW, molecular weight; detergent-soluble membrane fraction, an operational classification for material not sedimented during centrifugation of nonionic detergent-treated membranes at 1.9×10^5 g·min; detergent-insoluble membrane fraction, material sedimented during centrifugation of nonionic detergent-treated membranes at 1.9×10^5 g·min.

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

0091-7419/80/1404-0511$04.50

The binding of epidermal growth factor (EGF) to high-affinity receptors on responsive cells initiates a complex series of events that ultimately result in cellular proliferation [1]. The cell surface events that occur immediately following EGF binding have been the subject of intense study. Cell-bound fluorescent analogues of EGF become organized rapidly into clusters on the cell surface [2–4]. In an elegant electron microscopic study [5], Haigler et al observed a ferritin-EGF conjugate in clusters after its addition to cells, but prior to its internalization and incorporation into lysosomes. These authors also observed a sizable portion of bound ferritin-EGF in a clustered state in prefixed cells, suggesting that some unoccupied EGF receptors exist in clusters [5]. Clustering may be an important step in the mitogenic pathway, since an EGF derivative that induces cluster formation poorly is also a poor inducer of DNA synthesis [6].

Studies in this laboratory have focused on the direct determination of the fate of EGF receptors after EGF binding. We have identified the EGF receptors on murine and human cells by photoaffinity labeling [7], by a process termed direct linkage [8, 9], and by surface-specific iodination [10, 11]. We have followed the metabolic fate of photoaffinity-labeled EGF receptors and have demonstrated that they, like EGF itself, are internalized and degraded in lysosomes [12].

A more complete understanding of the biological function of the receptor requires its purification. With that goal in mind, we initiated attempts to solubilize the receptors found in high activity in membranes from A431 cells [13]. These receptors have two characteristic properties in nonionic detergent solutions: 1) a significant fraction of EGF receptors is totally refractory to solubilization in nonionic detergent solution; and 2) EGF receptors classified operationally as detergent soluble exhibit a remarkable tendency to exist in aggregates, rather than in a form resembling a monodisperse protein. These properties of EGF receptors differ from those described in a preliminary report by Carpenter [14].

EXPERIMENTAL PROCEDURES

Materials

Triton X-100, sodium dodecyl sulfate, sodium deoxycholate, Lubrol PX, and n-octyl glucoside were purchased from Sigma. Sepharose 6-B, manufactured by Pharmacia, was also purchased from Sigma. Polyethylene Glycol 6000 was from J. T. Baker. Sources of other materials are described elsewhere [9].

Procedures for cell culture, membrane preparation, EGF isolation and iodination, membrane-binding assays, and direct linkage complex formation and quantitation have been described elsewhere [9]. Most experiments utilized membranes prepared from A431 cells according to Procedure II [9]. Membranes were stored frozen in TS buffer prior to use.

Solubilization of EGF Binding Activity

Membranes were mixed with an aqueous buffered solution containing detergent and incubated for 30 min at 20°C. Detergent-insoluble material was removed by sedimentation at 12,800g for 15 min in a Brinkman microfuge. Material not sedimented under these conditions was operationally classified as "soluble." The validity of this operational classification is supported by two lines of evidence: 1) approximately 80% of the "soluble" membrane protein was included by Sepharose 6B (see Fig. 7); 2) neither the yield of solubilized ^{125}I-EGF binding activity nor its heterogeneity, as assessed by two independent physical methods (see Figs. 9 and 10), was altered when the preparation was first subjected to sedimentation at a higher centrifugal force (100,000g for 2 h).

When Triton X-100 was used for solubilization, it was added in the form of a 10% (v:v) solution in amounts corresponding to at least an 11-fold detergent:protein (w:w) excess, assuming a density of 1.10 for Triton X-100 [15]. In most experiments, the quantity rather than the concentration of detergent is specified, since the detergent-to-membrane-protein ratio is the more useful parameter in evaluating solubilization conditions at relatively high membrane concentrations [15]. Detergent concentrations are expressed as percentage by volume.

Determination of ^{125}I-EGF Binding by Polyethylene Glycol Precipitation

The procedure used was described originally for radioimmunoassay [16] and modified later for assay of detergent soluble insulin binding activity [17, 18]. The standard assay mixture contained the following in a total volume of 0.1 ml: ^{125}I-EGF at a final EGF concentration near the K_d for EGF binding; BSA (10 μg); membrane protein (0–10 μg); sodium phosphate (10 μmoles) or HEPES (1 μmole) at pH 7.4. Specific binding was the difference in ^{125}I-EGF bound in the presence and absence of 0.24 μmoles unlabeled EGF. The Triton X-100 extracted, particulate residue was assayed for ^{125}I-EGF binding activity after suspension in the original volume of detergent solution by vigorous mixing in the presence of one or two glass beads. Reaction mixtures were incubated at 20°C for 1 h, unless otherwise indicated. Bound ^{125}I-EGF was selectively precipitated by the sequential addition of 10 μl of a 2% (w:v) solution of bovine gamma globulin and 100 μl of a 25% (w:v) solution of Polyethylene Glycol 6000 (PEG) in 0.1 M Tris·HCl at pH 7.4. The assay mixtures were mixed vigorously and incubated on ice for 20 min. One hour of incubation after PEG addition did not affect the amount of radioactivity precipitated. The mixtures were filtered over Whatman GFC filters, the assay tubes were rinsed with 1–2 ml of 8% PEG in 0.1 M Tris·HCl, pH 7.4, and the filters were rinsed with 10 ml of the same solution.

When the formation of direct linkage complexes by the soluble extract and the particulate residue was examined (Fig. 6), the concentration of bovine gamma globulin was reduced by 20-fold to avoid overloading of the polyacrylamide gel with carrier protein. In the same experiment, the precipitate was then collected by sedimentation rather than by filtration.

Velocity Sedimentation and Gel Sepharose 6-B Chromatography Filtration

Velocity sedimentation was performed by established procedures [20]. Samples were sedimented through linear 5–20% sucrose gradients containing Triton X-100. Sedimentation of marker proteins varied linearly with their reported sedimentation coefficients [20]. Cuatrecasas has reported [18] that Triton X-100 does not affect the sedimentation coefficients of the marker proteins utilized. Gel chromatography was performed on Sepharose 6-B according to the manufacturer's instructions.

RESULTS

PEG Precipitation of ^{125}I-EGF Binding Activity From Detergent Extracts of A431 Membranes

The selective precipitation of large proteins by PEG provides a useful assay for measuring the binding of many small, and hence nonprecipitable, ligands to higher molecular weight receptor proteins in nonionic detergent solution [16–18]. PEG-induced precipitation is an excellent means of measuring ^{125}I-EGF binding in nonionic detergent-treated

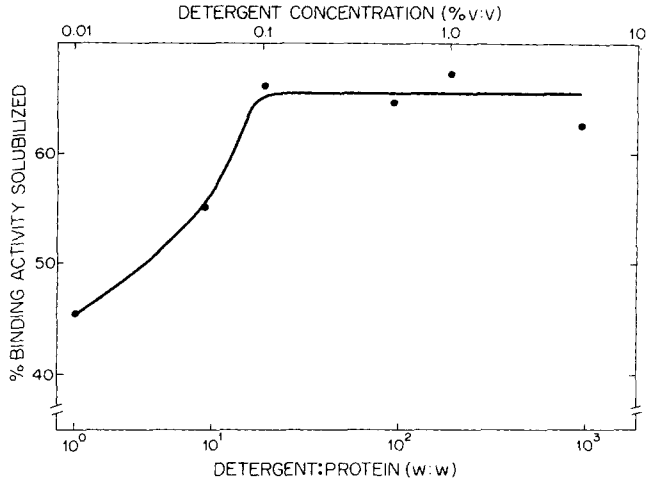

Fig. 1. Effects of varying Triton X-100 concentration on the solubility of EGF binding activity from A431 membranes. An aliquot of 150 μl of SP buffer containing 18 μg of A431 membrane protein was mixed with an equal volume of a solution containing sufficient Triton X-100 to give the stated final detergent concentration. After fractionation by sedimentation, as described in Experimental Procedures, ^{125}I-EGF binding was measured at 64 nM ^{125}I-EGF (1.8 × 10^9 cpm/nmole) by PEG precipitation. The total recovered binding activity did not vary systematically with detergent concentration and averaged 193 ± 18 fmoles EGF bound. The amount of binding activity solubilized is expressed as a percentage of the total recovered activity at a given detergent concentration.

A431 membranes. Over 80% of the ^{125}I-EGF specific binding activity present in untreated membrane suspensions was recovered with Triton X-100–treated A431 membrane suspensions. The PEG precipitation assay gives consistent results at concentrations of up to 5% Triton X-100 by volume. The recovery and extent of ^{125}I-EGF binding activity were constant when membranes at low protein concentration were treated with solutions containing from 0.1% to 5.0% Triton X-100 (Fig. 1).

A large portion (30–60%) of the recovered ^{125}I-EGF binding activity was sedimented by low-speed centrifugation. This was not reported by Carpenter [14] and was an unexpected result. The percentage of binding activity rendered soluble reached a maximum at a detergent protein ratio of 11:1 (w:w) (Fig. 1). ^{125}I-EGF binding by both the Triton-soluble and -insoluble fractions was rapid (Fig. 2) and was also linear with respect to protein concentration (Fig. 3).

The solubility of EGF binding activity was investigated further to determine if the insoluble fraction was an artifact of the conditions employed. A significant fraction of the EGF binding activity was operationally insoluble after treatment of A431 membranes with ionic (SDS, sodium deoxycholate) and other nonionic (Lubrol PX and N-octyl glucoside) detergents. An increase in the duration of incubation with detergent for up to 14 h did not result in more complete solubilization. The solubility of EGF binding activity in Triton X-100 solutions was not affected by the addition of 2 M urea, 3 M KCl, 20 mM EDTA, or by variations in pH between 6 and 10. Solubilization was not increased after alkylation of membranes with N-ethylmaleimide, indicating that the insoluble fraction is not the result of sulfhydryl oxidation. A similar fraction of the EGF binding activity in

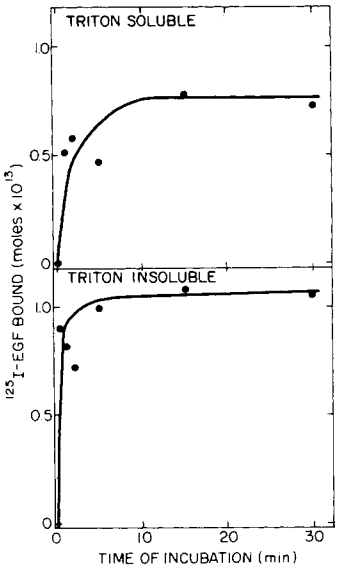

Fig. 2. Time course of ^{125}I-EGF binding to Triton X-100–treated A431 membranes. A membrane suspension (250 μg of protein) was treated with 2.8 mg of Triton X-100 in 1.85 ml of PBS. Fractionation into detergent-soluble and -insoluble fractions was achieved as described in Experimental Procedures. Eighty microliters of each fraction was assayed for EGF binding in the presence of 33 nM ^{125}I-EGF (0.57×10^9 cpm/nmole). At the times indicated, receptor-bound EGF was precipitated with PEG. The first sample was taken at 12 sec. All points are the average of duplicate determinations and are corrected for binding in the presence of 2.4 μM unlabeled EGF. Upper panel: ^{125}I-EGF binding to the Triton-soluble fraction of A431 membranes. Lower panel: ^{125}I-EGF binding to the Triton-insoluble fraction of A431 membranes.

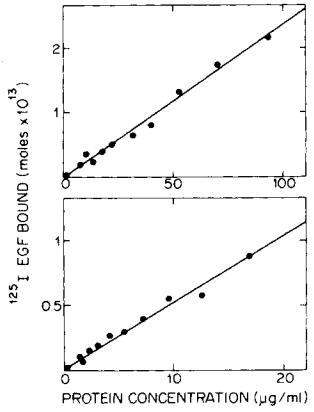

Fig. 3. PEG precipitation of ^{125}I-EGF binding activity at different concentrations of A431 membrane protein. A membrane suspension containing 160 μg of protein was treated with 5.5 mg of Triton X-100 in 1 ml of SP buffer and separated into the Triton-soluble and -insoluble fractions by sedimentation. Protein concentrations of 116 μg/ml for the soluble fraction and 21 μg/ml for the insoluble fraction were determined by a modified Lowry procedure [22]. Both fractions were serially diluted with SP buffer containing 0.4% Triton. An 80-μl aliquot of each dilution was assayed for binding at 35 nM ^{125}I-EGF (2.8×10^9 cpm/nmole). Top panel: ^{125}I-EGF binding to the Triton-soluble fraction. Bottom panel: ^{125}I-EGF binding to the Triton-insoluble fraction.

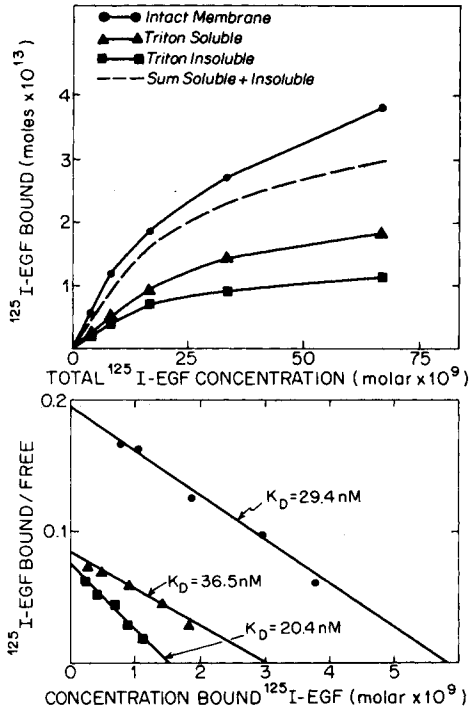

Fig. 4. Effects of EGF concentration on EGF binding to receptors on intact A431 membranes and to the Triton X-100–soluble and -insoluble fractions. Duplicate aliquots containing 90 μg of membrane protein were diluted to 1.85 ml with DBH containing 10 mg of Triton X-100. Samples of 80 μl of intact membranes or the detergent soluble or insoluble fractions were incubated for 1 h at 20°C with ^{125}I-EGF (0.9 × 10^9 cpm/nmole) at the indicated concentrations. Binding in samples containing intact membranes was determined by filtration [9]; binding in detergent-treated samples was determined by filtration after PEG precipitation. ●–●, ^{125}I-EGF binding by intact A431 membranes; ▲–▲, ^{125}I-EGF binding by the Triton-soluble fraction of A431 membranes; ■–■, ^{125}I-EGF binding by the Triton-insoluble fraction of A431 membranes; – – – –, sum of the binding by the soluble and insoluble fractions. Upper panel: ^{125}I-EGF bound versus ^{125}I-EGF concentration. Lower panel: Scatchard representation of the binding data.

membranes isolated from murine 3T3 or normal human fibroblast (HF-15) cell lines also was not soluble in Triton X-100 solutions. The insoluble fraction was evident when both frozen or freshly prepared membranes were examined. The insolubility of a significant portion of the EGF binding activity under such a wide variety of conditions provides evidence that EGF receptors exist partly in the form of supramolecular, detergent-insoluble, aggregates.

Affinities of Soluble and Insoluble Binding Activities for ^{125}I-EGF

The experiment described in Figure 4 demonstrated that the K_D of ^{125}I-EGF binding to receptors in the soluble fraction corresponded closely to the K_D determined with intact membranes; the K_D value for the insoluble fraction was slightly lower. This differs from the findings of Carpenter [14], who reported that the detergent-soluble fraction of EGF receptors displayed a significantly reduced affinity for ^{125}I-EGF. More recent experiments [26] performed in this laboratory have revealed that Scatchard plots of ^{125}I-EGF binding

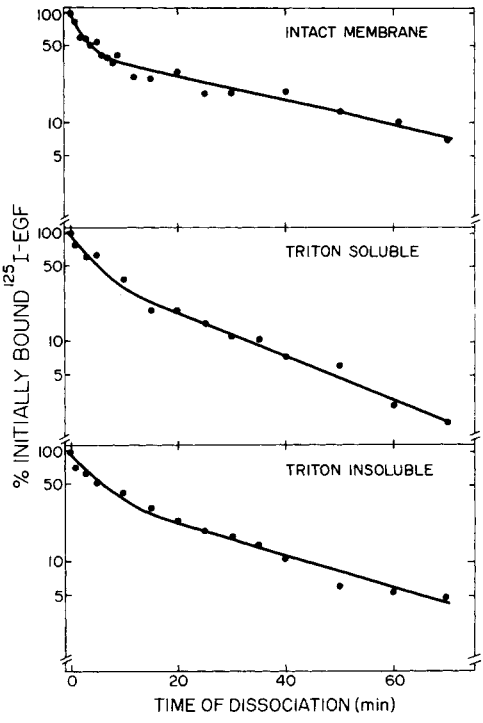

Fig. 5. Rate of dissociation of ^{125}I-EGF from EGF–receptor complexes in intact A431 membranes and in the Triton X-100–soluble or -insoluble fractions. Duplicate samples were prepared; each contained 530 μg of membrane protein in 1 ml of SP buffer and either 6 mg of Triton X-100 or no Triton X-100. The detergent-treated sample was fractionated by sedimentation as described in Experimental Procedures. The binding reaction was initiated by the addition of concentrated ^{125}I-EGF (0.60×10^9 cpm/nmole) to yield a final concentration of 35 nM and allowed to proceed for 1 h at 20°C. Immediately before establishing conditions for dissociation of the ^{125}I-EGF–receptor complex, duplicate aliquots of 25 μl were removed from each sample to serve as zero time controls. These aliquots were either filtered immediately or, where detergent-treated samples were involved, precipitated with PEG prior to filtration.

Dissociation was initiated by the addition of 3 volumes of SP buffer at 20°C, containing 1.1 μM unlabeled EGF. Aliquots of 100 μl were removed at the indicated times. All values are corrected for the amount of EGF remaining bound at 3 h (for the intact membranes) or 9 h (for detergent-treated fractions). Upper panel: Dissociation of the ^{125}I-EGF–EGF receptor complex on intact A431 membranes. The points at 1–10 min are the result of single determinations; all other points were assayed in duplicate. The amount of ^{125}I-EGF bound initially was 0.70 pmoles. Middle and lower panels: Dissociation from Triton-soluble or -insoluble fractions. All points are the average of duplicate determinations. The amounts of ^{125}I-EGF bound initially were 0.25 pmoles for the detergent-soluble fraction and 0.22 pmoles for the detergent-insoluble fraction.

to A431 membranes become curvilinear at lower EGF concentrations than those utilized in Figure 4. We cannot exclude the possibility that differences exist in high-affinity ^{125}I-EGF binding by the detergent-soluble and -insoluble fractions.

In the presence of a 100-fold excess of unlabeled EGF, the rate of dissociation of specifically bound ^{125}I-EGF from intact membranes is curvilinear (Fig. 5, top panel) and separable by extrapolation into two distinct components that dissociate with first-order kinetics. As shown in the middle and lower panels of Figure 5, both the detergent-soluble

and -insoluble fractions contain equivalent amounts of the rapidly and slowly dissociating components. This provides no evidence that the detergent-soluble and -insoluble forms of EGF receptors are distinguishable from EGF receptors in intact membranes on the basis of binding affinities.

Molecular Weights of EGF Receptors in the Detergent-Soluble and -Insoluble Fractions

Direct linkage of ^{125}I-EGF to its receptors is a useful technique for affinity labeling EGF receptors [8, 9]. The direct linkage complexes formed by receptor proteins in the detergent-soluble and -insoluble fractions were compared with those formed with intact membranes (Fig. 6). Both the soluble and insoluble fractions gave rise to direct linkage complexes of MW = 160,000, 145,000, and 115,000; these also are observed with A431 membranes [9].* This demonstrates that the same components of the EGF receptor are found in both the nonionic detergent-soluble and -insoluble fractions.

Characterization of the Physical Properties of the Soluble Binding Fraction

^{125}I-EGF binding activity of the Triton X-100–soluble fraction derived from different, independently isolated A431 membrane preparations was characterized by gel filtration chromatography and velocity sedimentation (see Fig. 9; Table I). Some detergent-solubilized cell surface receptors are homogeneous when fractionated by these techniques [18], but EGF receptors consistently behaved heterogeneously. The degree of heterogeneity varied in different membrane preparations: ten different preparations were analyzed by one, the other, or both techniques.

Gel filtration analyses. Figure 7 describes the gel filtration chromatography of a representative sample of Triton X-100–solubilized material on Sepharose 6-B. In five experiments, each performed with the detergent-soluble fraction from a different membrane preparation, between 62% and 84% of the binding activity recovered from the column (between 40% and 80% of that applied) was within the inclusion volume, but was distributed between several nonresolvable peaks. The most frequently observed peaks were centered at fractions 19–20 (corresponding to the void volume), 21–22, 25–26, and 31–32. The latter peak had the smallest Stokes radius (95 Å) of any regularly observed species and contained between 18% and 64% (mean = 40% ± 19%; median = 33%) of the recovered activity.

Sedimentation analyses. ^{125}I-EGF binding activity in the Triton X-100–soluble fraction also behaved heterogeneously during velocity sedimentation. In the six different membrane preparations analyzed, between 27% and 60% (mean = 44% ± 12%; medians = 42% and 46%) of the binding activity recovered from the gradient was in a peak sedimenting at 7S. Recoveries ranged from 33% to 100% of the activity applied. In some experiments, a minor resolvable peak was observed at 4S. The remaining activity sedimented at greater than 7S. A small amount of activity, which seldom exceeded 10% of that applied, was sometimes found pelleted after centrifugation. Figure 8 describes the results of the sedimentation experiment in which the proportion of EGF receptors behaved most like a monodisperse species. Even in this exceptional preparation, only 45% of the recovered activity sedimented at 7S. When the same preparation was analyzed by gel filtration as described in Figure 7, approximately 64% of the recovered activity was eluted from the Sepharose column at fractions 31–32. This suggests that the activity sedimenting at 7S and eluting

*The radioactive bands of mobility equal to or greater than BSA were not observed in samples not subjected to PEG precipitation [9]. These complexes are therefore an artifact of the precipitation process and do not represent specific ^{125}I-EGF–EGF receptor complexes.

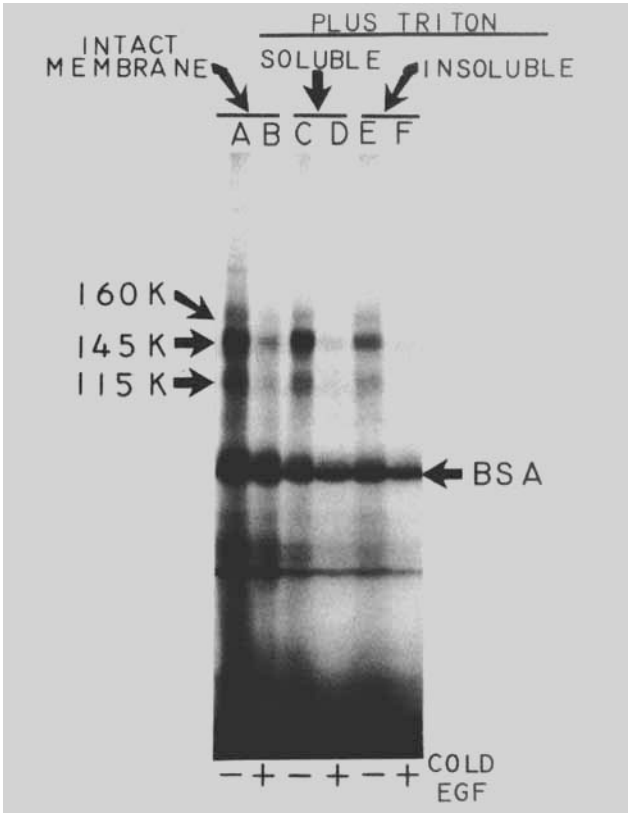

Fig. 6. Direct linkage of ^{125}I-EGF to its receptors on intact A431 membranes and in the detergent-soluble and -insoluble fractions. Duplicate samples of 0.49 ml of SP buffer containing 105 μg of membrane protein were prepared. One sample was treated with 3.3 mg of Triton X-100 in 30 μl of H_2O; the other was treated with H_2O as a control. The detergent-treated sample was then separated into soluble and insoluble fractions as described in Experimental Procedures. Aliquots of 80 μl of each sample were incubated with 56 nM ^{125}I-EGF (1.7×10^9 cpm/nmole) containing 2.4 μM unlabeled EGF where indicated. After 1 h of incubation at 20°C, receptor-bound EGF was precipitated with PEG and collected by sedimentation at 12,000g for 15 min in a Brinkman microfuge. The supernatant fractions were carefully removed and the pellets were dissolved in ESB. At this point the intact membrane sample contained 0.40 pmoles of specifically bound EGF; the Triton-soluble fraction, 0.17 pmoles; and the insoluble fraction, 0.16 pmoles. After electrophoresis and autoradiography, the percentage of specifically bound EGF present in the direct linkage complexes was determined to be 0.59% for the intact membrane, 1.4% for the Triton-soluble fraction, and 0.73% for the insoluble fraction. Lane A: direct linkage complexes formed by intact A431 membranes; lane B: as in A, but containing 2.4 μM unlabeled EGF; lane C: direct linkage complexes formed by the Triton-soluble fraction; lane D: as in C, but containing 2.4 μM unlabeled EGF; lane E: direct linkage complexes formed by the Triton-insoluble fraction; lane F: as in E, but containing 2.4 μM unlabeled EGF.

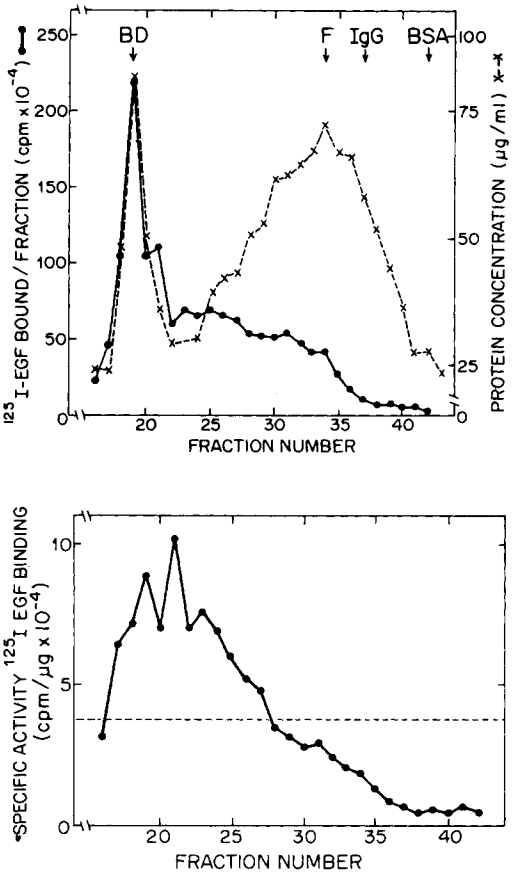

Fig. 7. Gel filtration chromatography of Triton X-100 solubilized EGF binding activity from A431 membranes. Membrane protein (2 mg, prepared according to Procedure II [9]) was treated with 0.2 ml of PBS containing 22 mg of Triton X-100 and separated into Triton-soluble and-insoluble fractions as described in Experimental Procedures. An aliquot (0.22 ml) of the detergent-soluble fraction was layered above a 1 × 25-cm column of Sepharose 6-B equilibrated with PBS plus 1% Triton. Fractions of 25 drops (0.3 ml) were collected. Ninely microliters of each fraction was incubated with 34 nM ^{125}I-EGF (3.1 × 10^9 cpm/nmole), and binding was measured by PEG precipitation. Total binding was corrected for background radioactivity (7463 cpm/fraction). Fifty-seven percent of the initial binding activity was recovered from the column. The slight peak running right behind the void volume (fraction 21) was reproducible. Markers were run on the same column under identical conditions. The elution volume of blue dextran (BD) was estimated visually, and that of equine ferritin (F) was determined spectrophotometrically by absorbance at 400 nM. The elution volumes of bovine immunoglobulin (IgG) and bovine serum albumin (BSA) were determined by gel electrophoretic analysis of the column fractions. Top panel: •—•, distribution of ^{125}I-EGF binding activity determined by the PEG precipitation assay. X———X, protein concentration determined by a modified Lowry procedure [25], using BSA as a standard. Bottom panel: •—•, specific activity of ^{125}I-EGF binding (cpm/μg protein); ————, specific activity of ^{125}I-EGF binding in the original detergent-soluble fraction.

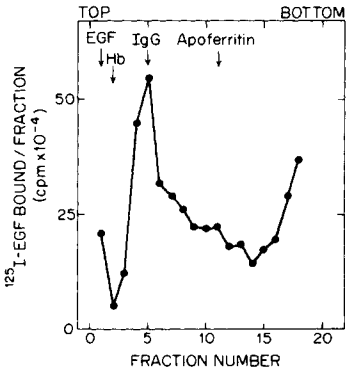

Fig. 8. Velocity sedimentation of Triton X-100 solubilized EGF binding activity from A431 membranes. The Triton X-100–soluble fraction from 1.25 mg of membrane protein (Procedure III [9]) was prepared exactly as described in the legend to Figure 7. A 0.1-ml aliquot of the Triton-soluble fraction was mixed with an equal volume of 1% bovine hemoglobin in PBS containing 10% (v:v) Triton X-100. The mixture was layered on top of a 5-ml, 5–20% (w:w) linear sucrose gradient in PBS containing 1% (v:v) Triton X-100, poured above a 0.2-ml cushion of 66% sucrose. Sedimentation was performed at 4°C in a Beckman SW-50.1 rotor for 14 h at 31,000 rpm. The tubes were punctured and 0.3 ml fractions (25 drops) were collected from the bottom. Ninety microliters of each fraction was incubated with 35 nM ^{125}I-EGF (1.13×10^9 cpm/nmole), and binding was measured after PEG precipitation and corrected for background radioactivity (5,800 cpm/fraction). Approximately 70% of the initial activity was recovered from the gradient. Sedimentation markers were fractionated on an identical gradient. The position of hemoglobin was estimated visually; the other markers had been lightly radioiodinated, and their positions were determined by gamma counting. The literature values for the sedimentation coefficients of the markers are as follows: EGF, 1.25S [23]; bovine hemoglobin (Hb), 4.1S [24]; rabbit immunoglobulin (IgG), 6.7S [24]; equine apoferritin (F), 17.6S [24].

from the Sepharose column at fractions 31–32 may be identical. It follows that the most rapidly sedimenting activity corresponds to that which was eluted with the void volume. When the material that was eluted in fractions 19–21 (the void volume) of the Sepharose column in Figure 7 was concentrated by vacuum dialysis and subjected to velocity sedimentation as described in Figure 8, approximately 65% of the recovered activity sedimented at greater than 19S, with 21% pelleting during centrifugation (data not shown).

The ^{125}I-EGF binding activity solubilized from 10 independent membrane preparations behaved heterogeneously when analyzed by the two fractionation techniques described (Table I). In experiments representative of the median range of behavior, between 30% and 40% of the recovered activity had behavior consistent with that of monodisperse EGF receptors. Since the yield of EGF-binding activity present in the detergent-soluble fraction has averaged approximately 60% in the 31 membrane preparations we have characterized to date, the percent of EGF binding activity behaving as a monodisperse entity is not likely to exceed 25% of the total activity (detergent-soluble plus detergent-insoluble EGF binding activity) recovered in the average preparation. Thus, the 95 Å, 7S form of EGF binding activity is not representative of the bulk of EGF binding activity present in detergent-treated A431 membrane preparations.

Numerous attempts were made to establish conditions to increase the proportion of EGF receptors rendered soluble in monodisperse form. Membranes were prepared from cells grown at different passage levels (10th to 40th from the culture initially received from

TABLE I. Variation in the Amount of Apparently Monodisperse EGF Binding Activity Solubilized From Different A431 Membrane Preparations*

	^{125}I-EGF binding (% of total recovered activity)	
Membrane preparation	95 Å peak	7S peak
A	–	27
B	–	36
C	57	–
7	30	–
8	18	–
9	33	–
17	64	36
29	–	60
30	–	42
31	–	55
Mean	40	44
SD	19	12

*Triton X-100–soluble fractions from several individual membrane preparations were fractionated by gel filtration chromatography and velocity sedimentation as described in Figure 7 and 8. The amount of apparently monodisperse, or lowest molecular weight, ^{125}I-EGF binding activity – ie, that which was eluted with an apparent Stokes radius of 95 Å or sedimented at 7S – was determined and expressed as a percentage of the total activity recovered.

G. Todaro), from cells grown with different batches of serum and at different serum concentrations (5–10%), and from cells grown to low or high density. In preliminary experiments in which these conditions were varied, only cell passage level had any effect on the extent of the detergent solubility of receptors.

Few solubilized proteins display the marked heterogeneity of the "soluble" EGF binding component. This heterogeneity indicates that some of the detergent-soluble binding activity exhibits a high degree of supramolecular organization. This heterogeneity is not due to a generalized aggregation of detergent-solubilized proteins, since ^{125}I-EGF binding activity fractionates distinctly from the bulk of solubilized protein during gel filtration chromatography (Fig. 7, top panel). The specific activity of ^{125}I-EGF binding is significantly enriched in the fractions eluting with and just after the void volume (Fig. 7, lower panel) of the Sepharose 6-B column.

Fractionation of Direct Linkage Complex-Forming Activity by Gel Filtration

The column fractions from the experiment illustrated in Figure 7 also were assayed for their ability to form direct linkage complexes. The radioactivity present in the major forms of the direct linkage complex displayed a heterogeneous distribution similar to that of the soluble binding activity (Fig. 9). While the elution patterns of ^{125}I-EGF binding and direct linkage complex formation activities were not strictly identical, they were qualitatively similar, suggesting that both activities copurify during gel-filtration chromatography. Direct linkage complex forming activity also fractionates with EGF binding activity during velocity sedimentation (data not shown). The copurification of direct linkage complex forming and EGF binding activities by both gel filtration chromatography and velocity sedimentation supports the view that direct linkage complexes are covalent complexes between ^{125}I-EGF and its receptors [8, 9].

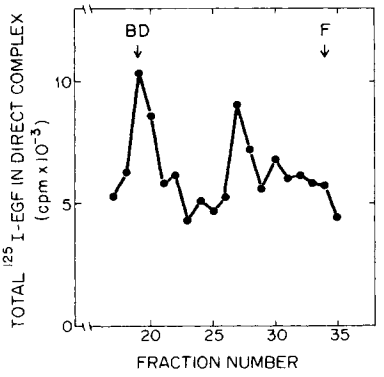

Fig. 9. Direct linkage complex formation by a Triton X-100 extract of A431 membranes subsequent to gel chromatography. Ninety microliters of the same column fractions produced in the experiment described in Figure 7 were incubated with 68 nM ^{125}I-EGF (3.1 X 10^9 cpm/nmole). After a 1 h incubation at 37°C, bound EGF was precipitated by PEG, collected by sedimentation as described in Figure 6, dissolved in ESB, and subjected to electrophoresis. The position of the direct linkage complexes was determined by autoradiography. Direct linkage EGF–EGF receptor complexes were formed with this particular membrane preparation at MW = 145,000 and 115,000 [9]. The radioactivity in each band was determined and corrected for the background radioactivity in an adjacent region of the same gel. Markers are blue dextran (BD) and equine ferritin (F).

DISCUSSION

Nearly half the EGF binding activity in membranes isolated from a variety of sources is not soluble in solutions containing up to a 1,000-fold weight excess of nonionic detergent. This may explain the low recovery (60%) of ^{125}I-EGF binding activity from Triton X-100–solubilized A431 membranes reported by Carpenter [14]. The EGF receptors solubilized by nonionic detergent solution display a marked tendency to exist as aggregates, and less than half the total EGF receptors can be solubilized in a monodisperse form. This aggregation could arise in several ways. It could be artifactual, similar to that exhibited by some detergent-solubilized membrane proteins when the detergent concentration is reduced to a value approaching the critical micelle concentration [15]. This is unlikely since most A431 membrane proteins were dispersed by the high concentrations of detergent (Fig. 7) employed in our experiments.

Covalent crosslinking by enzymes, such as the transglutaminase activity recently proposed to be involved in EGF interalization [25], could also lead to EGF receptor aggregation. If the receptor were covalently crosslinked into aggregates, it should engage in the formation of much larger forms of the direct linkage complex than those observed [9] and the yield of lower molecular weight complexes should be correspondingly reduced. However, the EGF receptors in the detergent-insoluble fraction of A431 membranes form direct linkage complexes with ^{125}I-EGF with molecular weights identical to the direct linkage complexes formed by receptors in the detergent-soluble fraction. Furthermore, the ratio of direct linkage complex formation to EGF binding in the detergent-insoluble and -soluble fractions is similar. Covalent crosslinking is thus not likely to be the origin of supramolecular forms of receptor observed in nonionic detergent extracts.

Haigler et al [5] have observed clusters of ferritin-EGF on the surfaces of prefixed A431 cells, an indication that clusters or aggregates of EGF receptors exist on the cell surface prior to EGF addition; these clusters may behave as aggregates of EGF receptors in nonionic detergent solution. Our data show that EGF receptors exist in the intact membrane in two forms: 1) an unclustered form, which is solubilized as a monodisperse protein; and 2) in clustered or aggregated forms. The highly aggregated and the disaggregated EGF receptors may display a number of different properties, including their biological potentials in vivo. Preliminary experiments indicate that the detergent-soluble and the highly aggregated detergent-insoluble forms of EGF receptors differ dramatically in the extent to which they accept phosphate [27] in the EGF-stimulated phosphorylation reaction described by Carpenter et al [17, 18].

The biological relevance of EGF receptor aggregation in cells not previously exposed to EGF is a speculative issue. A number of independent studies have established a relatively short half-life for surface-displayed EGF receptors on cultured cells [28–30]; these investigators base their observations on the rate of loss of EGF binding activity in murine or human cells exposed to inhibitors of protein [28–30] or RNA synthesis [29]. While it has not been established that steady-state turnover of receptors occurs by EGF-independent receptor clustering, internalization, and degradation by lysosomal proteases, this potential pathway is one of the most attractive explanations for the reported observations on this subject. We also have observed that EGF receptors are subject to down-regulation by other hormones – eg, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) [31, 32]. These observations suggest the existence of a common pathway for receptor turnover in which a variety of receptors are internalized in shared vesicles. This mechanism also was suggested on the basis of fluorescent microscopic observations made with differentially fluorescent ligands [21].

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 acknowledge the contributions of the following persons to this work: Cindy Blifeld, who assisted in the development of the PEG assay; Pamela Billings and Terry Lipari, who prepared membranes; Steve Ellis, who provided general technical assistance; and Betty Handy, who typed the manuscript.

REFERENCES

1. Carpenter G, Cohen S: Annu Rev Biochem 48:193–216, 1979.
2. Schechter Y, Schlessinger J, Jacobs S, Chang K, Cuatrecasas P: Proc Natl Acad Sci USA 75:2135–2139, 1978.
3. Schlessinger J, Schechter Y, Willingham MC, Pastan I: Proc Natl Acad Sci USA 75:2659–2663, 1978.
4. Haigler H, Ash JF, Singer SJ, Cohen S: Proc Natl Acad Sci USA 75:3317–3321, 1978.
5. Haigler HT, McKanna JA, Cohen S: J Cell Biol 81:382–395, 1979.
6. Schechter Y, Hernaez L, Schlessinger J, Cuatrecasas P: Nature 278:835–838, 1979.

7. Das M, Miyakawa T, Fox CF, Pruss RM, Aharonov A, Herschman H: Proc Natl Acad Sci USA 74:2790–2794, 1977.
8. Linsley PS, Blifeld C, Wrann M, Fox CF: Nature 278:745–748, 1979.
9. Linsley PS, Fox CF: J Supramol Struct 14:441–459, 1980.
10. Wrann M, Linsley PS, Fox CF: FEBS Lett 104:415–419, 1979.
11. Wrann M, Fox CF: J Biol Chem 254:8083–8086, 1979.
12. Das M, Fox CF: Proc Natl Acad Sci USA 75:2644–2648, 1978.
13. Fabricant RM, DeLarco JE, Todaro GJ: Proc Natl Acad Sci USA 74:565–569, 1977.
14. Carpenter G: Life Sci 24:1691–1698, 1979.
15. Helenius A, Simons K: Biochim Biophys Acta 415:29–79, 1975.
16. Desbuquois B, Averbach GD: J Clin Endocrinol 33:732–738, 1971.
17. Cuatrecasas P: Proc Natl Acad Sci USA 69:318–322, 1972.
18. Cuatrecasas P: J Biol Chem 247:1980–1991, 1972.
19. Carpenter G, King L Jr, Cohen S: Nature 276:409–410, 1978.
20. Martin RG, Ames BN: J Biol Chem 236:1372–1379, 1961.
21. Maxfield FR, Schlessinger J, Schechter Y, Pastan IH, Willingham MC: Cell 14:805–810, 1978.
22. Markwell MAK, Haas SM, Bieber LL, Tolbert NE: Anal Biochem 87:206–210, 1978.
23. Cohen S: J Biol Chem 237:1555–1562, 1962.
24. Sober JA (ed): "Handbook of Biochemistry," Ed 2. Cleveland: The Chemical Rubber Co, 1970.
25. Davies PJA, Davies DR, Levitzki A, Maxfield FR, Milhaud P, Willingham MC, Pastan IH: Nature 283:162–167, 1980.
26. Iwata KK, Williams RE, Fox CF: J Supramol Struct 9(Suppl 4):432, 1980.
27. Linsley PS, Fox CF: In Middlebrook J, Kohn L (eds): "Receptor-Mediated Internalization of Hormones and Toxins." New York, Random House, 1980.
28. Aharonov A, Pruss RM, Herschman HR: J Biol Chem 253:3970–3977, 1978.
29. Carpenter G: J Cell Physiol 99:101–110, 1979.
30. Fox CF, Wrann M, Linsley P, Vale R: J Supramol Struct 12:517–531, 1979.
31. Fox CF, Vale R, Peterson SW, Das M: In Sato G, Ross R (eds): "Hormones and Cell Culture." Conferences on Cell Proliferation Series. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1979, Vol 6, pp 143–157.
32. Wrann M, Fox CF, Ross R: Science 210:1363–1364, 1980.