The evidence suggests that the major 150,000 protein band is the receptor for EGF and is a substrate of the phosphorylation activity. Analysis of the affinity-purified human albumin; SDS, sodium dodecyl sulfate; HLB, hydrophobic balance. 

In this report, we present further characterization of the membranes with respect to kinetics of phosphorylation and dephosphorylation, reversibility of the EGF effect, and trypsin sensitivity. In addition, we present the following observations: (a) A-431 membranes may be solubilized with retention of EGF-enhanced phosphorylation activity as well as I251-EGF-binding activity, and (b) the EGF-receptor, the EGF-dependent kinase activity, and the substrate(s) for phosphorylation are co-purified with EGF-affinity chromatography.

EXPERIMENTAL PROCEDURES

Materials—Mouse EGF was isolated (13) and iodinated (2) by published procedures. [γ-32P]ATP was purchased from New England Nuclear. Histone (type II-A) was purchased from Sigma. Sephacryl S-300 was obtained from Pharmacia. Antiserum to EGF was prepared in rabbits using Freund’s adjuvant. Anti-EGF IgG was prepared as described (14). Trypsin (l-1-tosylamido-2-phenylethyl chloromethyl ketone) (TPCK)-treated) was a product of Worthington; soybean trypsin inhibitor was obtained from Sigma. Affi-Gel 10 was obtained from Bio-Rad. The detergents were commercial samples.

Growth of A-431 Cells and Preparation of Membrane Fraction—The A-431 cells were grown in 100-mm Falcon dishes or in roller bottles containing Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% calf serum (Flow Laboratories) and gentamycin (Microbiological Associates). Membranes were prepared by the procedures described by Thom et al. (15) and characterized as described previously (9). Protein was quantitated by the procedure of Bradford (16) using γ-globulin as a standard.

Standard Method for Membrane Solubilization—Aliquots of the A-431 membrane preparation were resuspended in 20 mM Hepes buffer, pH 7.4, containing 1% Triton X-100 and 10% glycerol. The final membrane protein concentration was 6 to 12 mg/ml. The suspensions were allowed to stand at room temperature for 20 min and the mixture was centrifuged at 100,000 × g for 60 min at 4°C. The supernatant fluid contained approximately 75% of the original membrane protein.

Binding of 125I-labeled EGF to A-431 Cell Membranes—The binding reaction was carried out as described (9) in a 200-μl reaction volume containing 20 mM Hepes buffer, pH 7.4, containing 1% Triton X-100 and 10% glycerol. The final membrane protein concentration was 6 to 12 mg/ml. The suspensions were allowed to stand at room temperature for 20 min and the mixture was centrifuged at 100,000 × g for 60 min at 4°C. The supernatant fluid contained approximately 75% of the original membrane protein.
cold 20 mM Hepes buffer, pH 7.4, containing 0.1% bovine serum albumin. The amount of radioactivity retained on each filter was determined by counting in a Nuclear Chicago Auto-Gamma spectrometer.

Binding of 125I-labeled EGF to Triton X-100-solubilized Membrane Preparations—Assays for soluble 125I-EGF-receptor complexes were determined as described previously (10). Reaction mixtures contained: (200 μl final volume) 20 mM Hepes buffer, pH 7.4, 0.1% bovine serum albumin, 0.2% Triton X-100, and 29 ng of 125I-labeled EGF. Solubilized receptor was added last to initiate the binding reaction. The assay mixtures were incubated at room temperature for 30 min and terminated by quickly adding 0.5 ml of 0.1% p-globulin in 0.1 M phosphate buffer, pH 7.4, and 0.5 μl of 20.4% polyethylene glycol 6000 (final concentration 8.5%). The mixtures were blended on a Vortex mixer and filtered on EHWP Millipore filters. Each filter was quickly washed with a total of 6 ml of 8.5% polyethylene glycol 6000 in 0.1 M phosphate buffer, pH 7.4. Nonspecific binding which was approximately 20% of the total binding was measured in replicate assay tubes containing a 100-fold molar excess of unlabeled EGF. The amount of radioactivity retained on each filter was determined by counting in a Nuclear Chicago spectrometer.

Standard Phosphorylation Assays—For intact membranes the reaction mixtures contained: A-431 membrane (40 to 80 μg of protein); Hepes buffer (20 mM, pH 7.4); MnCl₂ (1 mM); [γ-32P]ATP (15 μM, 6 to 12 10⁶ cpm); EGF (100 ng); and bovine serum albumin (7.5 μg) in a final volume of 60 μl. The reaction tubes were placed on ice and preincubated for 10 min in the presence or absence of EGF. The reaction was initiated by the addition of labeled ATP and incubation at 0°C was continued for the indicated times. The reaction was terminated by pipetting 50-μl aliquots onto squares (2 cm) of Whatman No. 3MM filter paper which were dropped immediately into a beaker of cold 10% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The filter papers were washed extensively with trichloroacetic acid/pyrophosphate solution at room temperature, extracted with alcohol and ether (5 min each) and dried, and the radioactivity was measured in a Nuclear Chicago gas flow counter.

For Triton X-100-solubilized membranes, the procedure was essentially identical, except the reaction mixtures were preincubated in the presence or absence of EGF for 10 min at room temperature and then chilled on ice for 10 min prior to initiating the reaction by the addition of the labeled ATP.

When the phosphorylation reaction was carried out for short periods of time (under 3 min), the reaction was stopped by the addition of 5 μl of 6 N HCl prior to the removal of the 50-μl aliquot for measurement of radioactivity.

Gel Electrophoresis and Autoradiography—The A-431 membrane components were separated by SDS-gel electrophoresis (7.5% acrylamide, unless otherwise noted) by the method of Laemmli (17). Molecular weight standards used for calibration of the gels were myosin, 200,000; β-galactosidase, 130,000; BSA, 68,000; ovalbumin, 43,000; carbonic anhydrase, 29,000; lysozyme, 14,300. The gels were fixed and stained with Coomassie blue as previously described (18).

The slab gels were dried under vacuum and autoradiography (1 to 10 days) was performed using Kodak RP Royal X-Omat film.

Preparation of EGF-Alf-Gel—Alf-Gel (an N-hydroxysuccinimide ester of a succinylated aminoalkyl Bio-Gel A support) was reacted with EGF according to the manufacturer's (Bio-Rad) directions using a ratio of 6 g of wet cake to 20 mg of EGF in 3 ml of 0.1 M NaHCO₃ buffer, pH 8. (In control experiments, the extent of coupling was estimated by including 125I-labeled EGF in the reaction mixture and it was found that approximately 30% of the EGF was incorporated into the gel.) The gel was then washed extensively using, successively, 4 liters of water, 2 liters each of 1 M NaCl, 0.1 M acetic acid, and 1 M urea, 4 liters of water, and finally, 2 liters of 20 mM Hepes buffer, pH 7.4.

RESULTS

Kinetic Properties of the Membrane Phosphorylation System—We have reported (9) that EGF binds to intact A-431 cell membranes, resulting in an enhanced phosphorylating activity of these membranes. Some of the kinetic parameters of these reactions were explored. In Fig. 1 is shown the time course of the phosphorylation reaction at 0°C in the presence and absence of EGF, with all reactants added simultaneously, i.e. the reaction was initiated by the addition of membranes to the appropriate incubation mixture. In the absence of EGF there appears to be a very rapid burst of phosphorylation within 10 s and then a slow increase in net phosphorylation. In the presence of EGF, the extent of phosphorylation is greater than that of the control at all times tested (10 s to 3 min) with the increase due to EGF becoming more apparent as the incubation progressed.

A precise kinetic interpretation of the effects of EGF on the rate of membrane phosphorylation is difficult since we must assume that a finite time is required for the binding of EGF and the activation of the phosphorylating system. We have therefore examined the effect of preincubation of the membranes with EGF for increasing periods of time on the initial rate of phosphorylation. Because of the rapid 10-s burst of phosphorylation in control membranes, it was necessary, for consistent results, to lengthen the incubation time to 30 s at 0°C. Under these conditions, preincubation of membranes with EGF for ½ to 5 min did not increase the initial rates of phosphorylation compared to that seen when all the reactants were added simultaneously as described in Fig. 1 (data not shown). These results attest to the rapidity of the EGF-receptor-kinase interactions.

The following experiments indicate that dephosphorylation reactions also occur with great rapidity, even at 0°C. Membranes were phosphorylated for 1 min using 32P-labeled ATP in the presence or absence of EGF; further incorporation of 32P was then prevented either by adding a 30-fold excess of unlabeled ATP to decrease the specific activity of the label, or by adding EDTA to inhibit phosphorylation by chelation of Mn²⁺. The phosphorylated membranes were then further incubated at 0°C and the 32P content of the membranes was measured. The results (Fig. 2) show that approximately half of the radioactivity initially incorporated into the membranes was lost in 2 min at 0°C after the addition of unlabeled ATP. The presence of EGF did not affect the rate of dephosphorylation. Results identical with those shown in Fig. 2 were obtained when EDTA was used in place of unlabeled ATP to inhibit 32P incorporation (data not shown). Since the dephosphorylation reactions were unaffected by the absence of Mn²⁺, it appears that the membrane kinase, which requires Mn²⁺ for activity, is not involved in the dephosphorylation system(s).

Fig. 1. Time course of the incorporation of 32P-phosphate from [γ-32P]ATP into intact A-431 membranes in the presence and absence of EGF. The standard phosphorylation assay was performed at 0°C as described under "Experimental Procedures," except that the reaction was initiated by the addition of membranes to the appropriate incubation mixture. ○—●, minus EGF; ○—○, plus EGF.

![Graph showing time course of incorporation of 32P-phosphate from [γ-32P]ATP into intact A-431 membranes in the presence and absence of EGF.]
EGF-Receptor-Kinase Interactions

The rapid dephosphorylation reaction apparently accounts for the observation (data not shown) that preincubation of the membranes with unlabeled ATP (15 μM) for 1 to 5 min prior to the addition of 32P-labeled ATP (final concentration 15 μM) for a 3-min incubation period did not decrease the extent of basal phosphorylation.

Reversibility of the EGF-induced Kinase Activation—The possibility was considered that the mechanism by which EGF activated the membrane kinase involved an irreversible or covalent modification of the kinase. Proteolytic activation of protein kinase from rat brain (19) and adenylate cyclase from rat ovaries (20) or cultured fibroblasts (21) has been reported. Therefore, the stability and reversibility of the EGF-induced kinase activation were examined. Membranes were "activated" by the addition of EGF and attempts were made to remove the membrane-bound EGF by extensive washing. As shown in Table I, Experiment 1, membranes treated with EGF remain in an activated state even after extensive washing.

A number of conclusions may be drawn from these experiments. EGF does not appear to cause the release from the membranes of either a soluble protein kinase or an activator of the kinase. The possibility is not excluded, however, that such molecules are released but sequestered within impermeant membrane vesicles. The capacity of exogenous histones to serve as a substrate for the kinase (9, 12) argues against sequestration.

The stability of the activated state of the membranes following washing could be due either to the continued presence of bound EGF or to an irreversible effect on the protein kinase itself. EGF-treated membranes were therefore exposed to antiserum directed against EGF. As shown in Table I, Experiment 2, exposure of EGF-activated membranes to anti-EGF serum resulted in a return of the phosphorylating capacity of the preparation to basal levels, whereas exposure to control antiserum had no effect. Further, addition of EGF to the washed antiserum-inactivated membrane preparation resulted in a reactivation of the phosphorylation system (Table I, Experiment 2, last column). We conclude from this experiment that the activation of the membrane kinase is reversible. The inactivation-reactivation phenomenon also argues against the possibility that EGF causes the release of a soluble protein kinase or kinase activator from these membranes.

In control experiments, the effects of varying washing procedures, with or without antiserum, on the dissociation of membrane-bound 125I-EGF were examined. The data in Table II show that, whereas washing in buffer only removed about 18% of the membrane-bound radioactivity, exposure to the antiserum reduced it to about 76%. This latter figure is probably...
low since some precipitated $^{125}$I-EGF-antibody complexes may have been retained in the pellet.

**Effects of Trypsin on EGF-binding and Phosphorylating Activities of A-431 Membranes**—Our studies have suggested that the membrane preparation contains at least three categories of components: the EGF receptor, the protein kinase, and substrates for the protein kinase (which may include the receptor or kinase). The sensitivity of each of these to tryptic digestion was examined in the following experiment. Membranes were digested with trypsin and assayed for (a) capacity to bind $^{125}$I-EGF, and (b) ability to phosphorylate either endogenous membrane components or exogenous histones in the presence or absence of EGF. The results, shown in Table III, suggest that of the components examined, the receptor for binding EGF was the least affected by tryptic digestion (only 30% inhibited). With regard to the phosphorylation reactions, assuming the results with added histones are a measure of total kinase activity, tryptic digestion inhibited approximately 40 to 60% of the kinase activity. The most trypsin-sensitive reaction was the phosphorylation of endogenous membrane components (about 80% inhibited), presumably reflecting the combined trypsin sensitivities of the membrane receptor, kinase, and substrates for phosphorylation.

**Solubilization of A-431 Membranes**—The solubilization of the $^{125}$I-EGF-binding activity of A-431 membranes by the use of Triton X-100 has been reported (10). We have examined detergent-solubilized membrane preparations for protein kinase activity and responsiveness to EGF. In preliminary experiments, it was found that over 50% of the endogenous basal kinase activity could be extracted with 1% Triton X-100; the inclusion of 10% glycerol in the detergent solution increased the yield to 70 to 90%. Attempts to stimulate the basal phosphorylation by the addition of EGF to solubilized preparations under the usual assay conditions (all incubations carried out at 0°C) were not successful. However, if EGF were allowed to react with the solubilized membrane preparation at room temperature prior to chilling the reaction tubes to 0°C for the phosphorylation assay, considerable activation of endogenous phosphorylation by EGF was detected. The results, depicted in Fig. 3, show that preincubation of Triton X-100 extracts with EGF at room temperature for approximately 5 min increased 2- to 3-fold the subsequent phosphorylation capacity of the membranes at 0°C. In other control experiments, preincubation of solubilized membranes with EGF at 0°C for 10 to 30 min had negligible effects on the phosphorylation capacity of the membranes.

A comparison of the effects of EGF on phosphorylation using intact and solubilized membranes is shown in Table IV. It may be seen that much (64%) of the basal activity and almost all (over 90%) of the EGF responsive activity are recoverable after solubilization.

The solubilization may be carried out at 0°C or room temperature; slightly higher yields of phosphorylating activity (about 10% higher) are observed using room temperature solubilization.

The Triton-glycerol extracts may be stored overnight at 0°C with retention of about 50 to 70% of the activity or at −70°C with retention of about 90% of the activity.

**Relation of Detergent to Solubilization of EGF-sensitive Phosphorylation**—In a series of exploratory experiments we treated A-431 membranes with a variety of detergents to examine their effectiveness for the solubilization of the EGF-sensitive phosphorylation system and to compare these results with those from other laboratories on the solubilization of membrane-associated enzymes.

The solubilizations and assays were performed exactly as described in Fig. 3, except for the detergent employed. Detergents which were approximately as effective as Triton X-100 (±30%) in solubilizing an EGF-enhanced phosphorylation activity were: Tween 21, Triton CF 54, Triton N 101, Triton X-114, Triton CF 10, and Triton N 111. These detergents have HLH numbers ranging from 12.4 to 14. Detergents having higher or lower HLH numbers were much less effective (less than 20% as efficient as Triton X-100). These include (with HLH numbers in parentheses): Tween 80 (15), Tween 20 (16.7), Tween 81 (10), and Atlas G-1086 (10.2). The major exception was Brij 30, which, although having an HLH number of 8.7, is effective as Triton X-100.

### Table III

**EGF-Insensitive and Phosphorylating Activities of A-431 Membranes**

<table>
<thead>
<tr>
<th>Conditions of incubation</th>
<th>$^{125}$I-EGF bound</th>
<th>Membrane protein</th>
<th>Histone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGF+EGF</td>
<td>EGF−EGF</td>
<td>+EGF</td>
</tr>
<tr>
<td>Minus trypsin</td>
<td></td>
<td></td>
<td>10,400</td>
</tr>
<tr>
<td>Plus trypsin</td>
<td>7,380</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>Plus trypsin and trypsin inhibitor</td>
<td>10,500</td>
<td>650</td>
<td>1,520</td>
</tr>
</tbody>
</table>

* $^{32}$P incorporation into histone was calculated by subtracting the incorporation in the absence of histone from the total incorporation in the presence of histone.
TABLE IV

Comparison of effects of EGF on phosphorylation in intact and solubilized membranes

Aliquots of A-431 membranes (400 µg of protein) were suspended in 200 µl of either (a) 20 mM Hapes, pH 7.4, (b) 20 mM Hapes containing 1% Triton X-100, or (c) 20 mM Hapes containing 1% Triton X-100 and 10% glycerol. The suspensions were allowed to stand at room temperature for 20 min and the mixtures containing detergent were centrifuged at 100,000 g for 60 min. Aliquots (15 µl) of the intact membranes or detergent-solubilized supernatant were preincubated with EGF for 10 min at room temperature prior to the assay for phosphorylation (10 min at 0°C, see "Experimental Procedures").

<table>
<thead>
<tr>
<th>Preparation</th>
<th>32P incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact membranes</td>
<td>1,050</td>
</tr>
<tr>
<td>Solubilized (1% Triton)</td>
<td>510</td>
</tr>
<tr>
<td>Solubilized (1% Triton, 10% glycerol)</td>
<td>670</td>
</tr>
</tbody>
</table>

The results presented in Table IV show that 2-fold higher concentrations of EGF (8 × 10−8 M) are required to achieve maximal phosphorylation in solubilized preparations, and half-maximal stimulation occurred at approximately 2 × 10−8 M.

The extent of phosphorylation of the solubilized A-431 membrane preparation was nearly proportional to membrane protein concentration in the range of 15 to 60 µg/tube (data not shown).

Gel Filtration of the Triton X-100-solubilized Membrane

In Fig. 5, we compare membrane proteins phosphorylated in the presence and absence of EGF, using both intact and solubilized membrane preparations, by SDS-acrylamide electrophoresis and autoradiography. It may be seen that the major phosphorylated component of both preparations is a doublet whose components have molecular weights of approximately 170,000 and 150,000. The phosphorylation of this doublet is clearly stimulated in both intact and solubilized membranes by the addition of EGF. The phosphorylation of other membrane components, however, is less apparent in the solubilized preparations. While added histones are phosphorylated in both membrane preparations, the solubilized preparation is much less efficient in this regard as compared to intact membranes (data not shown). As expected, the Coomassie blue patterns of the intact membranes and solubilized preparation were similar (Fig. 5).

With regard to the dependence of the extent of phosphorylation on the concentration of EGF, we have reported (9) that, using intact membranes, maximal stimulation of membrane phosphorylation occurred in the presence of approximately 3 to 4 × 10−8 M EGF and that half-maximal stimulation occurred at approximately 1 × 10−7 M. The results presented in Fig. 6 show that 2-fold higher concentrations of EGF (8 × 10−8 M) are required to achieve maximal phosphorylation in solubilized preparations, and half-maximal stimulation occurred at approximately 2 × 10−7 M.

The extent of phosphorylation of the solubilized A-431 membranes, by SDS-acrylamide electrophoresis and autoradiography, as described under "Experimental Procedures." The reaction was stopped by the addition of 50 µl of Laemmili SDS sample buffer (17). The mixtures were boiled for 5 min and subjected to SDS-gel electrophoresis. Coomassie blue staining, and autoradiography, as described under "Experimental Procedures." BSA was present in all samples. B and C, Coomassie blue stain of membrane components from intact (B) and solubilized (C) preparations; D to G, autoradiography of membrane components phosphorylated in absence (D and F) and presence (E and G) of EGF. D and E, intact membranes; F and G, solubilized membranes.
System— Attempts were made to fractionate the phosphorylating and EGF-binding activity of the solubilized membranes by gel filtration using Sephacryl S-300. The results of these efforts are shown in Fig. 7. From the data we conclude that: (a) most of the $^{125}$I-EGF-binding activity and EGF-stimulated phosphorylation activity elute together in a volume which suggests a molecular size larger than a Triton X-100 micelle ($M_r = 90,000$) or catalase ($M_r = 232,000$), but smaller than ferritin ($M_r = 440,000$); (b) most of the EGF-independent phosphorylating activity elutes with the excluded peak; and (c) most of the solubilized membrane protein distributes from the excluded peak to the position of Triton micelles.

SDS-acrylamide electrophoretic analysis of Fractions 10 to 14 in Fig. 7 indicated that each fraction, although detectably different from the other with regard to the relative amounts of Coomassie blue-stained bands, still contained a very complex mixture of proteins (data not shown).

Since both the EGF-binding and EGF-stimulated phosphorylation activities in the Triton extracts penetrated the Sephacryl matrix, these experiments provide additional evidence that "solubilization" had indeed occurred.

**Affinity Purification of EGF Receptor-Kinase Complex**—In view of the complex composition of the Triton extracts and the limited quantities of membrane available, we have attempted to purify and resolve the EGF receptor and EGF-sensitive phosphorylation activities by affinity chromatography with EGF covalently linked to Affi-Gel. Since high affinity ligand binding and elution were involved, we chose to effect the separation by a "batch" procedure where temperature and time could be closely controlled. In brief, Triton extracts of A-431 membranes were stirred with EGF-Affi-Gel and the gel was washed exhaustively and then incubated with a high concentration of EGF to effect elution. Since the EGF present in the eluate precluded $^{125}$I-EGF-binding studies, only phosphorylation activity was examined. A typical result is shown in Table V. It may be seen that adsorption to the EGF-Affi-Gel resulted in extracts which apparently retained basal phosphorylation capacity but had lost much of the EGF-stimulated activity. Elution of the EGF-Affi-Gel with EGF resulted in the recovery of about 37% of the original phosphorylation activity.

**Table V**

<table>
<thead>
<tr>
<th>Sample</th>
<th>-EGF</th>
<th>+EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original extract</td>
<td>750</td>
<td>1,970</td>
</tr>
<tr>
<td>Nonadsorbed supernatant</td>
<td>650</td>
<td>890</td>
</tr>
<tr>
<td>EGF-eluate</td>
<td></td>
<td>720</td>
</tr>
</tbody>
</table>

**EGF-Affi-Gel purification of protein kinase activity by elution with EGF**

A-431 membranes (360 $\mu$g of protein) were solubilized in 200 $\mu$l of 20 mM Hepes buffer, pH 7.4, containing 1% Triton X-100 and 10% glycerol as described under "Experimental Procedures." After centrifugation, 150 $\mu$l of the solubilized preparation were added to a Sephacryl S-300 column (0.9 $\times$ 31.5 cm) equilibrated with the membrane-solubilizing buffer. One-milliliter fractions were collected at a flow rate of 0.15 ml/min. Aliquots (35 $\mu$l) of the fractions were assayed for their capacity to bind $^{125}$I-EGF as described under "Experimental Procedures." Aliquots (15 $\mu$l) of the same fractions were assayed for their endogenous phosphorylation capacity in the presence and absence of EGF by the standard method described under "Experimental Procedures." Vertical bars, the $^{125}$I-EGF specifically bound in the aliquots taken from each fraction; $\times$—$\times$, protein content; $\bullet$—$\bullet$, $^{32}$P incorporated in the absence of EGF; $\circ$—$\circ$, $^{32}$P incorporated in the presence of EGF.
The elution of material from the EGF-Affi-Gel by EGF presumably was dependent upon competition for the solubilized adsorbed receptor. Since assaying for receptor activity in the presence of such large quantities of EGF was not possible, we examined alternate procedures to elute the receptor, or kinase, or both, from the affinity gel. It was found that dilute solutions of ammonia or ethanolamine were capable of eluting both the EGF receptor and EGF-dependent phosphorylating activity from the EGF-Affi-Gel beads. In brief, Triton extracts of A-431 membranes were stirred with EGF-Affi-Gel and the gel was washed exhaustively and incubated with 5 mM ethanolamine, pH 9.7, to effect elution. The resulting eluate was neutralized and assayed for $^{125}$I-labeled EGF binding and for endogenous phosphorylation capacity in the presence and absence of EGF. The details of the procedure and a typical result are shown in Table VI.

Approximately 39% of the original $^{125}$I-EGF-binding activity was recovered in the ethanolamine eluate. With regard to the endogenous phosphorylation activity eluted from the gel, it should be noted that a stimulatory effect of EGF was detectable. In many repeated experiments, the stimulatory effect of EGF ranged from 2.3- to 4-fold. The recovery of phosphorylating activity in the ethanolamine eluate (Table VI, third column) appears low (14%) relative to the recovery of binding activity (39%). This may reflect the fact that not all of the kinase activity is associated with the receptor or that some of the substrates for phosphorylation (endogenous membrane protein) are also removed by the purification procedure (see next section).

In the following experiments, we compared: (a) the composition of the original solubilized membrane preparation with the material eluted from the EGF-Affi-Gel (by SDS-gel electrophoresis and Coomassie blue staining), and (b) the nature of the components phosphorylated in the original extract with those phosphorylated in the material eluted from the EGF-Affi-Gel (by SDS-gel electrophoresis and autoradiography).

TABLE VI

<table>
<thead>
<tr>
<th>Sample</th>
<th>$^{32}$P incorporated</th>
<th>$^{125}$I-EGF binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original extract</td>
<td>710</td>
<td>370,000</td>
</tr>
<tr>
<td>Nonadsorbed supernatant</td>
<td>730</td>
<td>80,000</td>
</tr>
<tr>
<td>Ethanolamine eluate</td>
<td>153</td>
<td>144,000</td>
</tr>
</tbody>
</table>

FIG. 8. Electrophoresis and autoradiography of solubilized A-431 membranes purified by affinity chromatography. A-431 membranes (10 mg of protein) were solubilized in 800 ml of 20 mM Hepes buffer, pH 7.4, containing 1% Triton X-100 and 10% glycerol as described under "Experimental Procedures." After centrifugation, aliquots (200 to 400 ml) were adsorbed to EGF-Affi-Gel beads and the beads were washed as described in the legend to Table V. Elution of the adsorbed material by either EGF or ethanolamine was carried out as described in the legends to Tables V and VI, respectively. Aliquots of the original extract, the nonadsorbed material, and the EGF or ethanolamine eluates were subjected to the standard phosphorylation procedures in the presence and absence of EGF for 10 min at 0°C as described under "Experimental Procedures." The reactions were stopped and analyzed by SDS-gel electrophoresis, Coomassie blue staining, and autoradiography as described in the legend to Fig. 5 and under "Experimental Procedures." A to D, Coomassie blue stain of original extract (A), nonadsorbed material (B), EGF eluate (C), and ethanolamine eluate (D); E to K, autoradiography of membrane components phosphorylated in the absence (E, G, and J) and presence (F, H, I, and K) of EGF. E and F, original extract; G and H, nonadsorbed material; I, EGF eluate; J and K, ethanolamine eluate.
The elutions were carried out either by the EGF procedure or by the ethanolamine procedure.

We conclude from the Coomassie blue data shown in Fig. 8 that: (a) the composition of the nonadsorbed material in the Triton extracts (Lane B) is almost identical with that of the original extract (Lane A); (b) elution of the gel with either EGF or ethanolamine results in identical patterns (Lanes C and D) consisting of one major 150,000 band. Trace quantities of other bands (not apparent in the photograph) are detectable on the original gels at the M, = 170,000, 130,000, and 50,000 to 60,000 regions. Thus, we have achieved this method a very considerable purification. Due to the difficulties in measuring the trace quantities of protein available, especially in the presence of Triton, precise purification ratios were not obtained. However, amino acid analysis of the original extract and the ethanolamine eluate (after acid hydrolysis) indicated that approximately 1% of the original protein was recovered.

We conclude from the autoradiography data shown in Fig. 8 (Lanes E to K) that under the conditions employed, the major phosphorylated component in the original Triton extract was a doublet in the M, = 150,000 to 170,000 region (Lanes E and F). In the non-adsorbed Triton supernatant, the patterns were similar except that the EGF-stimulated M, = 150,000 to 170,000 components had decreased (Lanes G and H). In both the EGF and ethanolamine eluates, only this doublet was detected, with the major radioactive band (M, = 150,000) corresponding with the major Coomassie blue-staining material. The stimulatory effect of adding EGF to the ethanolamine eluate during the phosphorylation is clearly seen (compare Lanes J and K).

It should be recalled that the material subjected to SDS-electrophoresis (original extract, nonadsorbed supernatant, ethanolamine eluate (Fig. 8, Lanes E, G, and J, respectively)) were replicates of the fractions examined for 125I-EGF-binding and phosphorylation capacity (Table VI). To summarize, the ethanolamine eluate possesses both 125I-EGF-binding capacity and EGF-stimulated phosphorylation activity. SDS-electrophoretic analysis of this fraction shows the presence of one major M, = 150,000 protein band and a few trace bands. The M, = 150,000 protein is a substrate of the phosphorylation reaction.

DISCUSSION

We and others have reported previously that the plasma membrane of A-431 cells possesses an extraordinarily high concentration of receptors for EGF, approximately 2 to 3 X 10^6/cell (5, 23). Membranes may be prepared from these cells which not only retain binding activity toward EGF, but also possess a cyclic AMP-independent protein kinase activity which is increased in the presence of the hormone. The kinase activity requires the presence of Mn++ or Mg++, and the phosphorylation is directed primarily toward threonine residues present in specific proteins of the membrane (9).

Some of the kinetic parameters of this reaction were examined in this report. The data indicate that: (a) activation of the membrane-associated kinase activity by EGF is a very rapid process, even at 0°C; (b) dephosphorylation reactions in the membrane also occur with great rapidity, but are unaffected by the presence of EGF; (c) EGF does not appear to cause the release from the membrane of either a soluble protein kinase or activator of the kinase; and (d) the EGF-induced activation of the membrane kinase may be reversed by removal of the hormone from the membrane by anti-EGF IgG.

We have reported previously that EGF-stimulated kinase activity of A-431 membranes is not removed by extraction of the membranes with a variety of solutions (high salt, urea), suggesting that the kinase and receptor are intrinsic membrane proteins (12). The solubilization of both activities only by those detergents which have been shown to solubilize membrane proteins in other systems (22), supports this view.

It is not known why preincubation of the solubilized membrane preparation with EGF at room temperature for several minutes is required to elicit a kinase response. This temperature-dependent incubation period is not necessary when intact membranes are used. Although part of the answer may be accounted for by the observation that solubilized membranes can bind 2- to 3-fold more EGF at room temperature compared to 0°C (10), this does not appear to account entirely for the observed effect.

We have reported, using intact membranes, that the major EGF-stimulated phosphorylated component is a doublet migrating in the M, = 150,000 to 170,000 region upon SDS-gel electrophoresis (12). An EGF-dependent phosphorylation of a number of other bands, as well as added histones, also has been noted. Upon solubilization with Triton, the data (Fig. 5) show that, with intact membranes, EGF stimulates the phosphorylation of M, = 150,000 to 170,000 doublet. The phosphorylation of other membrane components, as well as that of histones, although detectable, is much less prominent than in intact membranes.

The gel filtration data (Fig. 7) indicate that EGF-binding activity and EGF-stimulated kinase activity have similar molecular dimensions (molecular weights on the order of 300,000 to 400,000). It is not known whether or not the kinase activity, or the receptor, or both, are present as Triton micelles. If both are indeed in micelles, the efficiency of the EGF-stimulated phosphorylation reaction suggests they are in the same micelle and were probably, therefore, associated together in the intact membrane. This latter supposition takes on additional support from the data obtained by adsorption of the solubilized membrane preparation with EGF-Affi-Gel.

The adsorption to the affinity gel of EGF-binding activity together with EGF-stimulated phosphorylation activity suggests an inherent association of these two activities, since only the receptor could be presumed to have an affinity for the EGF-Affi-Gel. SDS-gel electrophoresis and Coomassie blue staining of the material eluted from the EGF-Affi-Gel, by either EGF or ethanolamine, show only one major protein band at M, = 150,000 with a trace band in the M, = 170,000 region and several smaller trace bands. We have calculated, from the binding capacity of intact membranes (9), assuming a molecular weight of 150,000 for the receptor, that receptor protein represents approximately 0.3% of the membrane protein. It is very tempting to presume that the M, = 150,000 band itself represents the receptor which is phosphorylated in the presence of EGF and that one of the minor bands is actually the kinase, but this conclusion must be verified by more rigorous experiments. The possibility that 32P is incorporated into a trace protein in the M, = 150,000 region has not been excluded. The acetylcholine receptor has been shown (24) to be a substrate for endogenous membrane kinase activity; in this instance, the level of receptor phosphorylation is decreased by the presence of cholinergic ligands. In other systems (25, 26), autophosphorylation of protein kinases has been observed. Several groups have attempted to identify the EGF receptor by covalent cross-linking to 125I-labeled EGF. Hock et al. (27) report receptor molecular weights of 180,000 and 160,000 in placenta. Wran and Fox (28) and Das et al. (29) report a molecular weight for the EGF receptor of 175,000 in A-431 cells and 190,000 in 3T3 cells, respectively.

We have assumed, based on previous data (9) which clearly indicate different heat sensitivities of the receptor and the kinase, that two separate entities are involved. It is conceiv-
able that the receptor and the kinase activities are present in the same molecule. Data regarding these questions, as well as the question of the mechanism by which EGF induces kinase activity (for example, whether EGF is phosphorylated as an intermediate), are not yet available.

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REFERENCES