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Controlled Proteolysis of EGF Receptors: Evidence for Transmembrane Distribution of the EGF Binding and Phosphate Acceptor Sites

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A small quantity of the ^{125}I-EGF (epidermal growth factor) bound specifically to EGF receptors on the human epidermoid carcinoma cell line A431 associates covalently. The direct linkage complex formed migrates during gel electrophoresis as a single diffuse band of MW = 160,000–170,000. In contrast, direct linkage complexes of 160,000, 145,000, and 115,000 daltons are formed when EGF is incubated with membranes isolated from these cells; these arise from EGF receptor modification during membrane isolation. None of these modifications affected the affinity of the EGF binding site for ^{125}I-EGF.

The electrophoretic mobilities of the MW = 160,000 and 145,000 direct linkage complexes were similar to those of the major ^{32}Pi-labeled products of the EGF-stimulated phosphorylation reaction described by Carpenter et al [Nature 276:409–410, 1978], indicating that proteolytic fragments of EGF receptors are the major phosphate acceptors in this reaction. EGF receptors on intact A431 cells accepted phosphate effectively from γ-^{32}Pi-ATP only when the cells were permeabilized with lysolecithin. This shows that the EGF binding and phosphate acceptor sites lie on opposing faces of the membrane. When the 145,000 dalton form of receptor is labeled with EGF or ^{32}Pi and the labeled peptides subjected to tryptic hydrolysis under identical conditions, all phosphate is lost from high molecular weight products under conditions where the EGF-receptor covalent complex is converted largely to a 115,000 dalton form. This suggests that the phosphate acceptor site lies on the cytoplasmic side of the membrane on a region of receptor extending 30,000 daltons from the 115,000 dalton fragment containing the EGF binding site.

Key words: protein phosphorylation, permeabilized cells, EGF receptors – transmembrane distribution, fragmentation by trypsin, phosphate acceptor site

Epidermal growth factor (EGF) initiates its action by binding to specific cell surface receptors on responsive cells [1]. Cell-bound EGF is internalized in endocytic vesicles, which ultimately fuse with lysosomes [2], where it is proteolytically degraded into its component

Abbreviations: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle medium; DBH, DMEM minus $NaHCO_3$, containing 10 mM HEPES at pH 7.4 and 0.1% bovine serum albumin; ST buffer, 0.25 M sucrose in 10 mM Tris · HCl at pH 7.4; BSA, bovine serum albumin; ESB, electrophoresis sample buffer (3% sodium dodecyl sulfate and 10% glycerol in 63 mM Tris · HCl at pH 6.8); MW, molecular weight (M_r); buffer A, 0.15 M sucrose, 0.08 M KCl, 5 mM potassium phosphate, 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$ in 35 mM HEPES at pH 7.4.

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amino acids [3]. Since a loss of cellular binding capacity for EGF (known as EGF-induced receptor down regulation) accompanies the internalization and degradation of EGF, cell surface receptors for EGF were presumed to be processed similarly [3]. Studies performed in this laboratory to determine the metabolic fate of a photoaffinity crosslinked ^{125}I-EGF–EGF receptor complex demonstrated that EGF receptor down regulation proceeds by receptor internalization and degradation by lysosomal proteases [4, 5]. The identical EGF dose responses for EGF receptor down regulation and induction of DNA synthesis led to the hypothesis that proteolytic processing of EGF or its receptor is an obligatory step in the mechanism of hormone action [6].

Recently, we [7, 8] and Baker et al [9] observed that a small portion of the ^{125}I-EGF specifically bound to its receptor on a variety of cell types undergoes a spontaneous and irreversible "direct linkage" to its receptors. Because of the potential utility of direct linkage as an affinity labeling technique for EGF receptors, we conducted a detailed investigation of the process. The complexes formed with isolated membranes were generally of lower molecular weight and more heterogeneous than the complexes formed with intact cells [10]. This showed that EGF receptors are proteolytically processed during membrane isolation. Here we have exploited these EGF–EGF receptor complexes to demonstrate that a phosphate acceptor site on a segment of the EGF receptor has a transmembrane distribution from the EGF binding site.

EXPERIMENTAL PROCEDURES

Materials

Trypsin (type XII: 2X crystallized) and soybean trypsin inhibitor (type II S: 1 mg inhibits 0.9 mg trypsin) were from Sigma. TPCK-treated trypsin (254 units/mg) was obtained from Worthington. γ-^{32}Pi-ATP was obtained from Amersham. Sources of other materials are indicated elsewhere [10].

Methods

Procedures for cell culture, membrane isolation, EGF preparation and iodination, gel electrophoresis, EGF binding, and direct linkage complex formation are described elsewhere [10]. EGF-stimulated phosphorylation of membrane proteins was accomplished by the procedure of Carpenter et al [11, 12].

RESULTS

Comparison of the EGF–EGF Receptor Direct Linkage Complexes Formed With Intact A431 Cells and Isolated Membranes

The direct linkage complex formed with intact A431 cells migrated as a diffuse band of MW = 160,000–170,000 (Fig. 1, lane A), and this complex was not readily degraded to specific products by trypsin added either before (lane B) or after (lane C) direct linkage complex formation. When A431 cells were first scraped from their substratum, and then incubated with ^{125}I-EGF, they did not give rise to the diffuse MW = 160,000–170,000 band in gel electrophoresis. Instead, they yielded direct linkage complexes of MW = 160,000, 145,000, and 115,000 (Figs. 1 and 2).

The MW = 145,000 complex formed with isolated membranes (lane D) was also the major radiolabeled product visible on gels when cells labeled by direct linkage were scraped from their substratum in ST buffer at 4°C 2 min prior to their solubilization (data not

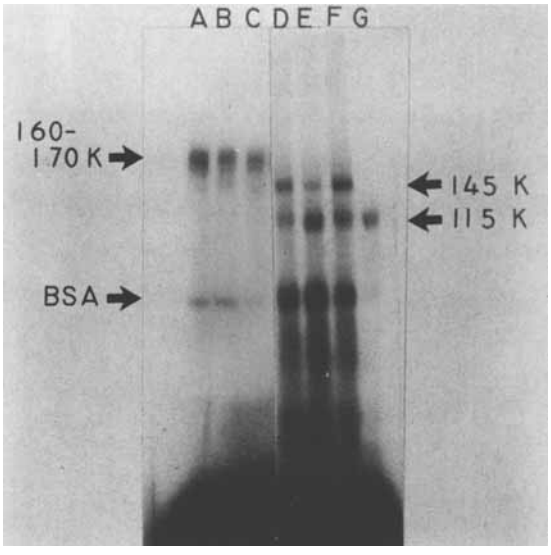

Fig. 1. Characterization of the direct linkage complexes. Direct linkage complex formation with intact cells. Intact monolayers of A431 cells in 28 cm^2 plastic dishes were incubated at 20°C for 1 h with 33.3 nM ^{125}I-EGF (2.6×10^9 cpm/nmole) to form direct linkage complexes between EGF and receptor. Where indicated, cultures were treated before or after ^{125}I-EGF binding for 30 min at 4°C with 5 ml of a solution containing 0.25 mg/ml of trypsin in DMEM. The cultures were washed thoroughly after trypsin treatment. The culture that was treated with trypsin before labeling was incubated for 15 min at 4°C with 2.5 mg/ml of soybean trypsin inhibitor in DMEM and washed again prior to the initiation of the binding reaction. Each culture was solubilized in ESB as described in Experimental Procedures. An 80 μl aliquot was subjected to electrophoresis.

Direct linkage complex formation with membranes. Membranes (6 μg of protein, prepared according to Procedure II [10]) were suspended in 0.1 ml of DMEM containing, where indicated, 25 μg of trypsin and 250 μg of soybean trypsin inhibitor. The mixtures were incubated at 4°C for 30 min and sedimented for 15 min at 12,800g. The pellets were washed with DMEM. Each membrane pellet was suspended in 0.1 ml of DBH containing 2.25 mg/ml of soybean trypsin inhibitor and 130 nM ^{125}I-EGF. After a 1 h incubation at 20°C, the membranes were collected by sedimentation and the pellets were suspended in 0.1 ml of DMEM with or without 0.25 mg/ml of trypsin. After a 30 min incubation at 4°C, the membranes were collected by sedimentation and dissolved in ESB, and the proteins were subjected to electrophoresis. All samples were resolved on the same acrylamide slab. Intervening lanes were cut from the photograph of the autoradiogram. The radioactivity in the 68,000 dalton region is a complex between ^{125}I-EGF and BSA; its formation was not inhibited by unlabeled EGF and was not investigated further.

Lane A: direct linkage complex formed in intact A431 cells; lane B: as in A, except that the culture was treated with trypsin prior to ^{125}I-EGF binding; lane C: as in A, except that culture was treated with trypsin after ^{125}I-EGF binding.

Lane D: direct linkage complexes formed with isolated membranes (experimental conditions were analogous to those in lane A); lane E: as in D, with trypsin treatment prior to ^{125}I-EGF binding; lane F: as in E, except that trypsin was mixed with soybean trypsin inhibitor prior to its addition to membranes; lane G: as in D, except that trypsin treatment took place after ^{125}I-EGF binding.

shown). This finding shows that the EGF receptors were degraded during or shortly after the scraping procedure. The activity(ies) responsible for the production of a lower molecular weight, MW = 145,000 form of the direct linkage complex was not inhibited by inclusion of 10% fetal calf serum in the isotonic buffer solution into which the cells were scraped from the tissue culture dish. The physiological role of this uncharacterized proteolytic

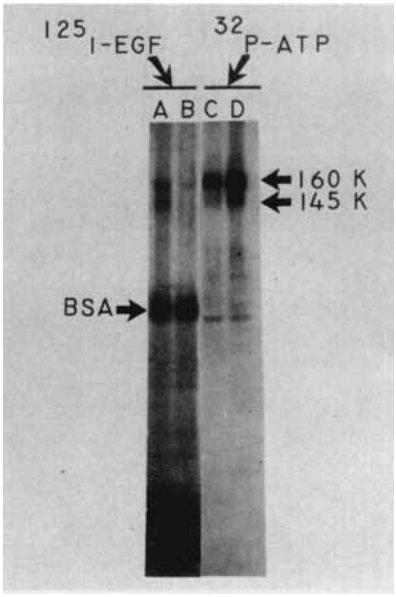

Fig. 2. Comparison of phosphorylated membrane proteins with the direct linkage complexes. Direct linkage complex formation was assayed using 0.1 ml reaction mixtures containing 8.75 μg of membrane protein, 139 nM ^{125}I-EGF (1.6 × 10^9 cpm/nmole), and where indicated, 2.38 μM unlabeled EGF. After a 90 min incubation at 37°C, the membranes were collected by sedimentation and washed once. The final membrane pellets were dissolved in 0.1 ml of ESB, and 40 μl of each sample was subjected to electrophoresis.

The membrane proteins labeled by the rapid, EGF stimulated phosphorylation described by Carpenter et al [11] were compared with those that link directly to ^{125}I-EGF. Membrane protein (Preparation III [10], 28 μg) was suspended in 38 μl of a solution containing 2.8 μmoles of HEPES, pH 7.4, 0.28 μmoles of MnCl, 19 μg of BSA, and 8.3 pmoles of unlabeled EGF, where indicated. The samples were incubated for 10 min at 0°C prior to the addition of 12 μCi of γ-^{32}Pi-ATP (1 mCi/ml, 3,000 Ci/mmole). After a 2.25 min incubation at 0°C, the reactions were terminated by the addition of an equal volume of twice concentrated ESB. Forty microliters of each sample was subjected to electrophoresis.

Since the phosphorylated and iodinated samples required different exposure times to achieve comparable grain densities on x-ray film, the dried gel was cut in half; each isotope was exposed separately and a final composite figure was derived from photographs of each half. The amount of radioactivity present in the MW = 145,000 and 160,000 regions of each lane was determined as described in Experimental Procedures for ^{125}I or by Cerenkov counting for ^{32}Pi. Of the ^{125}I-EGF present as direct linkage complexes, 63% was present as the MW = 160,000 dalton product. Of the total ^{32}Pi in the MW = 160,000 and 145,000 regions, 75% was present as the MW = 160,000 product. EGF stimulated by 2.6-fold the ^{32}Pi incorporated in the MW = 160,000 region and by 2.2-fold that incorporated in the MW = 145,000 region.

Direct linkage, lane A: formation of direct linkage complexes (MW = 160,000 and 145,000). – A complex between ^{125}I-EGF and BSA was formed in this experiment; unlabeled EGF did not block formation of this complex; lane B: as in A, plus unlabeled EGF.

Phosphorylation, lane C: phosphorylation of membrane proteins in the absence of EGF; lane D: EGF-stimulated phosphorylation of membrane proteins; as in C, plus EGF.

activity is unclear, although the product of its action, the MW = 145,000 component, is sometimes detected in small amounts in populations of mitogenically competent murine cells [10]. For the purpose of this discussion we refer to this activity as "scraping protease."

The MW = 115,000 direct linkage complex is formed by further modification of higher molecular weight forms of EGF receptors or EGF–EGF receptor direct linkage complexes. The receptor portion of the 145,000 dalton complex is sensitive to trypsin added either

before (lane E) or after (lane G) direct linkage complex formation; the direct linkage complex product is a radiolabeled MW = 115,000 fragment that may be identical to that which accumulates physiologically on cells incubated in the presence of chloroquine [5, 7]. The MW = 145,000 complex formed with isolated membranes was rapidly degraded at one-eighth the trypsin-receptor ratio that produced much less hydrolysis of the MW = 160,000–170,000 complex formed on intact cells (lanes B and C). This finding suggests that the protease-sensitive site that gives rise to the 115,000 dalton form of the EGF receptor direct linkage complex becomes exposed during membrane isolation. Protease treatment is widely used for determining the vectorial orientation of membrane proteins [13]. The insensitivity of EGF receptors to trypsin in intact cells relative to membranes provides evidence that a trypsin-sensitive portion of the receptor extends into the cytoplasm. The cleavage site for the scraping protease is also likely to be located on the cytoplasmic portion of the receptor, since broken cell preparations do not contain activities that degrade EGF receptors on intact cells (data not shown).

The endogenous proteases responsible for the lower molecular weight direct linkage complexes act prior to rather than after direct linkage complex formation. The time courses for formation of the MW = 160,000, 145,000, and 115,000 direct linkage complexes in membranes show that the corresponding EGF receptor precursors bind EGF and form direct linkage complexes independently [10]. The data in Figure 1 provide additional documentation for the retention of EGF binding activity by fragments of EGF receptors [5, 10]. The MW = 115,000 fragment is formed when membranes are treated with trypsin before EGF addition (lane E). Since a concentration of soybean trypsin inhibitor sufficient to inhibit the action of any residual trypsin (lane F) was present at the time of EGF addition, the component giving rise to the MW = 115,000 form of the direct linkage complex existed prior to EGF addition.

The K_d for ^{125}I-EGF binding was similar when measured with intact cells or with isolated membranes displaying varying amounts of the MW = 160,000, 145,000, and 115,000 forms of the direct linkage complex (Table I). The similar K_d values for receptors on intact cells or isolated membranes show that no major changes in the affinity for ^{125}I-EGF are produced by the action(s) of the endogenous protease activity that produces the MW = 145,000 component or that which produces the MW = 115,000 component.

Correlations in the Electrophoretic Behavior of Direct Linkage Complexes and Proteins Labeled by EGF Stimulated Phosphorylation

The electrophoretic behavior of direct linkage complexes formed with A431 membranes was compared with that of the proteins phosphorylated in the EGF-stimulated reaction described by Carpenter et al [11]. The proteins phosphorylated specifically in response to EGF addition migrate during gel electrophoresis with mobilities similar to those of the MW = 160,000 and 145,000 fragments of the direct linkage complex (Fig. 2). The slight differences in mobility between the fragments of the direct linkage complex and the phosphorylated proteins (Fig. 3) can be accounted for by the 6,000 daltons contributed by EGF. While co-migration is not a rigorous criterion for protein identity, the fact that two related fragments of the EGF–EGF receptor direct linkage complex each co-migrate with a protein that is phosphorylated in the EGF-stimulated reaction provides strong evidence that the EGF receptor is itself the major protein phosphorylated in this reaction.

Differential Trypsin Sensitivity of Radiophosphate-Labeled and Direct Labeled EGF Receptors

Two identical samples of membranes were first radiolabeled – one sample by direct linkage of ^{125}I-EGF to receptor, the second, by EGF-induced phosphorylation – and then

TABLE I. Effects of Endogenous EGF Receptor Proteolysis on ^{125}I-EGF Binding*

System	Distribution of radioactivity in direct linkage complexes products[a]	K_d
Intact cells		
Experiment 1	160 K (61%) 145 K (39%)	23 nM
Experiment 2	160 K (45%) 145 K (55%)	23 nM
Membranes		
Experiment 1	145 K[b]	28 nM
Experiment 2	160 K (23%) 145 K (41%) 115 K (37%)	29 nM

*^{125}I-EGF binding was determined at several EGF concentrations with intact cells and isolated membranes giving rise to differing amounts of the main direct linkage complexes. Binding to intact cells was compared using two separate populations of A431 cells obtained from G. Todaro. ^{125}I-EGF (3.5×10^9 cpm/nmole) was added at concentrations ranging from 0.8 nM–100 nM. Specific binding was determined as described in Experimental Procedures and plotted according to Scatchard [17]. The K_d of binding was determined from the slopes of the line best fitting the data. Identical cultures were incubated with 16.7 nM ^{125}I-EGF in order to form the direct linkage complexes. After electrophoresis, the relative amounts of radioactivity in the indicated regions of the gel were determined as described elsewhere [10].
[a]Measurements of binding and direct linkage complex formation with isolated membranes were performed in an analagous fashion, as described elsewhere [10].
[b]No data are available for the relative distribution of other direct linkage products in this experiment; the 145 K fragment was predominant.

treated with varying amounts of trypsin (Fig. 3). The radiophosphorylated protein migrating in the band corresponding to the 145,000 dalton form of the direct linkage complex was far more sensitive to tryptic digestion than were the direct linkage labeled receptors (lanes B and G). This could result from structural modifications of receptor caused by covalent coupling with phosphate or EGF. This difference also could be related to the finding that there are forms of the receptor having differential phosphate acceptor activities [14]; these forms could have inherently different susceptibilities to tryptic digestion.

The direct linkage labeled receptor yielded tryptic digestion products not observed with radiophosphate-labeled receptor. Digestion of the direct linkage complex first gives rise to the MW = 115,000 component, and then to a broad band of radiolabeled material centering on MW = 85,000 (lanes B and E). No products in either of these molecular weight ranges were observed following tryptic digestion of the radiophosphate-labeled protein corresponding to the 145,000 dalton direct linkage complex (lanes G–J). Several explanations can be advanced to account for this behavior. First, the phosphate may reside on a low molecular weight fragment of the protein produced by trypsin treatment. A phosphate-labeled band migrating at approximately 30,000 daltons was detected after trypsin treatment (lane G), but this could have arisen from the digestion of other phosphate-labeled, higher molecular weight components. Second, tryptic digestion of membranes might accelerate cleavage of the bond linking phosphate to receptor.

Inaccessability of EGF-Stimulated Protein Kinase in Intact A431 Cells to Externally Added γ-^{32}Pi-ATP

EGF receptors in intact A431 cells were tested for their ability to accept ^{32}Pi from γ-^{32}Pi-labeled ATP (Fig. 4, lanes A and B). Negligible radioactivity was incorporated migrating with bands at the electrophoretic mobility of EGF receptors. When the permeability of

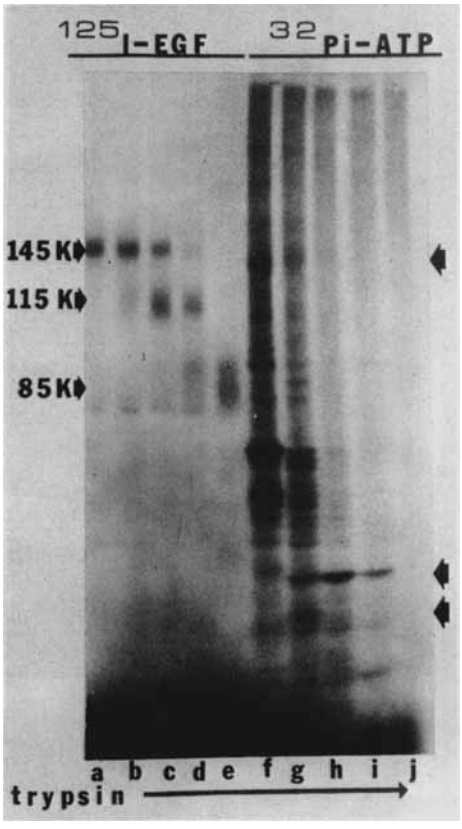

Fig. 3. Trypsin treatment of direct linkage complexes and phosphorylated membrane proteins. Two identical samples of A431 membranes (360 μg protein) were incubated for 1 h at 37°C with 104 nM labeled (1.2×10^9 cpm/nmole) or unlabeled EGF in a final volume of 0.1 ml of ST buffer. Both mixtures were then diluted with an equal volume of a solution of 0.1 M HEPES at pH 7.4, containing 0.4 M NaCl, 10 mM MnCl, and 0.05% BSA. The sample containing unlabeled EGF was mixed with 100 μCi of γ-^{32}Pi-labeled ATP (1 mCi/ml, 3,000 Ci/mmole); the sample containing ^{125}I-EGF was treated with H_2O as a control. The phosphorylation reaction proceeded for 1.5 min on ice. EDTA was then added to both samples to a final concentration of 13 nM. The membranes were washed by sedimentation through a sucrose shelf as described elsewhere [10], washed with DMEM, and suspended in 110 μl of DMEM. Identical 20 μl aliquots of both the direct linkage labeled (lanes A–E) and radiophosphate-labeled (lanes F–J) membranes were then mixed with 5 μl of TPCK treated trypsin in DMEM. After an additional 30 min at 0°C, each sample was mixed with an equal volume of twice-concentrated ESB. Aliquots (20 μl) of each sample were then subjected to electrophoresis. The arrows on the left-hand side of the figure denote the positions of the MW = 145,000 direct linkage complex and its major tryptic products. The uppermost arrow on the right-hand side denotes the position of the major protein phosphorylated in an EGF-induced reaction (MW = 145,000). The bottom two arrows denote low molecular weight labeled proteins which accumulate during trypsin treatment (MW = 30,000–40,000); the appearance of the lower of these follows most closely the loss of radioactivity from the MW = 145,000 ^{32}Pi-labeled species. Lanes A, F: no trypsin; lanes B, G: 0.0125 μg trypsin; lanes C, H: 0.125 μg trypsin; lanes D, I: 1.25 μg trypsin; lanes E, J: 12.5 μg trypsin.

cells to radiolabeled nucleotide was enhanced by mild lysolecithin treatment [15], EGF-stimulated incorporation of ^{32}Pi into components in the molecular weight range of EGF receptors was increased dramatically (lanes C and D). The ability of these components to ac-

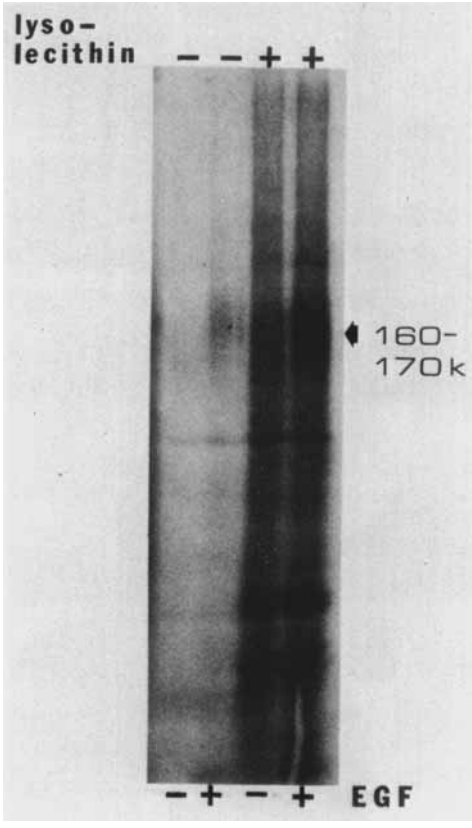

Fig. 4. Phosphorylation of EGF receptors in intact A431 cells. Identical 28 cm^2 monolayer cultures of A431 cells were treated with (lanes B and D) or without (lanes A and C) 67 nM unlabeled EGF in DBH. After incubation for 1 h at 23°C, the cells were washed twice with ice-cold buffer A [15], and two cultures (lanes C and D) were permeabilized as described by Miller et al [15]. After the addition of 0.1 mCi/well of γ-^{32}Pi-ATP (1 mCi/ml, 3,000 Ci/mmole), incubation was continued at 4°C for an additional 15 min. All cultures were then solubilized in ESB, and 20 μl aliquots were subjected to electrophoresis [10].

cept phosphate from ATP after disruption of the cellular permeability barrier is consistent with the localization of the phosphate acceptor site on a region of receptor not exposed to the extracellular space.

DISCUSSION

Direct linkage complex formation was exploited to make two observations on the structural properties of EGF receptors: 1) The EGF receptor is highly susceptible to proteolytic digestion during membrane isolation. Some proteolytic fragments observed with isolated membranes are also present in populations of mitogenically stimulated cells [16]; the ability of defined fragments of the EGF receptor to interact with EGF should be considered in the assessment of the proposed role of receptor processing during mitogenic activation [6]. 2) Two molecular weight forms of the EGF receptor comigrate during gel electrophoresis with the major proteins phosphorylated in the rapid, EGF-stimulated reaction described by Carpenter et al [11]. This indicates that the EGF receptor is itself the major phosphate acceptor in this reaction.

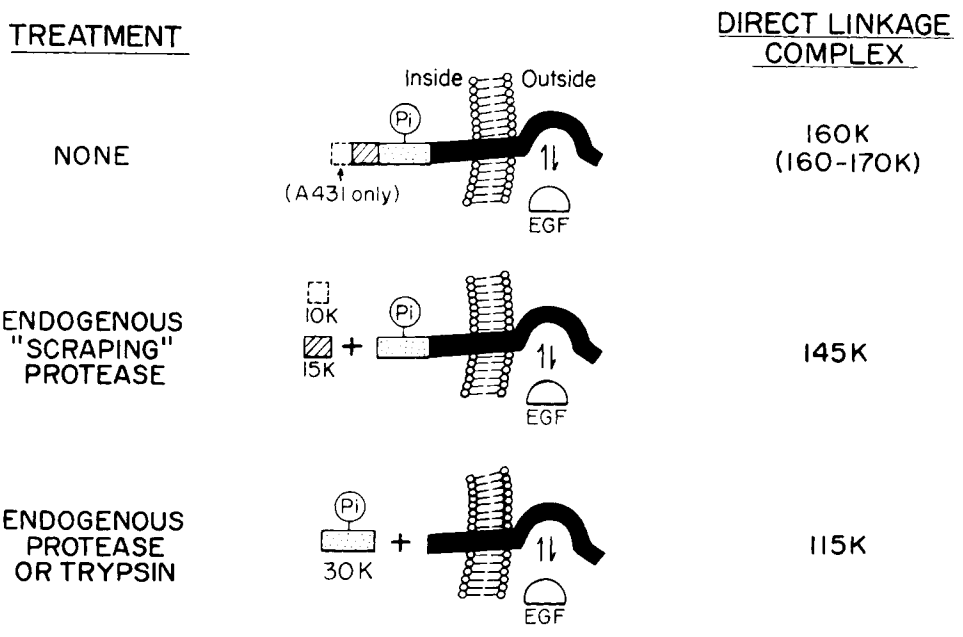

Fig. 5. A structural model of the EGF receptor. The data presented in Figure 1 and Table I demonstrate the sequential, endogenous proteolytic modifications of EGF receptors which do not appreciably affect their ability to bind ^{125}I-EGF. The EGF receptors in intact A431 cells form a direct linkage complex which migrates in gel electrophoresis as a diffuse band of MW = 160,000–170,000. This diffuse band migrates slightly behind the much sharper band of the MW = 160,000 complex formed with intact HF-15 [9] or 3T3 cells [P.S. Linsley and C.F. Fox, unpublished data] or that formed with isolated membranes from A431 cells (Fig. 2). A MW = 145,000 component of the direct linkage complex is formed by the action of an endogenous "scraping protease" activated when cells are scraped from their substratum. This is the prominent form of receptor in membranes isolated by the procedures employed. In some membrane preparations a direct linkage complex of MW = 115,000 is formed. Its electrophoretic behavior is identical to that of the complex generated by treatment of isolated membranes (but not intact cells) with trypsin. Both the MW = 145,000 and 115,000 direct linkage complexes remain membrane bound.

Since the MW = 145,000 component of the direct linkage complex comigrates during gel electrophoresis with a form of the EGF receptor that is phosphorylated in response to EGF addition (Fig. 2), the site of phosphorylation is not removed by the scraping protease. The ^{32}Pi incorporated in both the MW = 160,000 and 145,000 forms of the receptor in response to EGF addition is completely removed when membranes are treated with trypsin under conditions that lead to the formation of the MW = 115,000 form of the direct linkage complex (Fig. 3). The phosphorylated site on the receptor is therefore likely to be located on a MW = 30,000 region released by trypsin treatment.

The EGF receptor in intact A431 cells is relatively insensitive to exogenously added trypsin. Broken cell preparations also are ineffective in cleaving the MW = 160,000–170,000 direct linkage complex formed on intact cell monolayers (data not shown). The MW = 145,000 component of the direct linkage complex present in isolated membranes is converted totally to the MW = 115,000 component by concentrations of trypsin that do not degrade the MW = 160,000–170,000 complex present on intact cells to discrete products (Fig. 1 and data not shown). The cleavage sites for both the scraping protease and trypsin are therefore most likely displayed on the cytoplasmic side of the membrane.

A hypothetical structural model of EGF receptors based on our observations is presented in Figure 5. This model identifies three distinct sites exposed as the permeability barrier of the cell is disrupted. Two of these are protease-sensitive sites (see Fig. 1), and the third site accepts phosphate from ATP in a reaction stimulated by EGF addition (see Figs. 2 and 4).

One of the protease-sensitive sites gives rise to a 145,000 dalton direct linkage complex of EGF and receptor; the second, to a 115,000 dalton form. The text of the legend of Figure 5 presents the rationale for the presence of the phosphate acceptor site on the region of receptor that is lost when the MW = 145,000 ^{125}I-EGF–receptor complex is cleaved to yield the 115,000 dalton complex.

Cohen et al have observed a gel electrophoretic doublet in the 150,000–170,000 MW range as the major products of EGF-induced phosphorylation in detergent extracts of A431 cells; they concluded that this is likely to be the EGF receptor [18]. These bands probably are analogous to the 145,000 and 160,000 dalton direct linkage ^{125}I-EGF–receptor complexes and the corresponding products that we observe after EGF-induced radiophosphorylation (Fig. 4). Additionally, the major product of EGF-induced radiophosphorylation in lysolecithin "permeabilized" cells is in the 160,000–170,000 dalton range (Fig. 4). We conclude that the higher molecular weight form of the direct linkage EGF receptor complex and its radiophosphate-labeled counterpart is the intact receptor, and that the lower molecular weight forms are artifacts produced by endogenous protease activity. The endogenous activities released during membrane isolation degrade receptor to fragments that accept ^{125}I-EGF to produce 145,000 and 115,000 dalton labeled complexes. Treatment of the 145,000 dalton ^{125}I-EGF–receptor complex with trypsin also gives rise to a 115,000 dalton fragment, indicating a receptor region that contains a site or sites sensitive to hydrolysis by either trypsin or endogenous protease activity.

Proteolytic digestion of EGF receptors labeled by direct linkage with ^{125}I-EGF or by phosphorylation with γ-^{32}Pi-ATP proceeds at different rates. These different rates of digestion could result from alterations in receptor conformation induced by covalent addition of EGF, phosphate, or both. Alternatively, the root of these differences in rate of digestion by protease may lie in the observation that the nonionic detergent-soluble and -insoluble forms of receptor can be labeled by direct linkage with ^{125}I-EGF, whereas the detergent-solubilized receptors are readily labeled by EGF-induced phosphorylation and the detergent-insoluble receptors are not [14]. The direct linkage labeled receptors may therefore consist of a mixed population, some of which are readily sensitive to proteolytic digestion and some of which are not.

When A431 membranes are first radiophosphate labeled in the presence of EGF and then treated with trypsin, products of lower molecular weight arise as the phosphate-labeled receptor bands disappear. One of these products has a molecular weight of approximately 30,000. This might be the fragment released during the stepwise degradation of the 145,000 dalton direct linkage labeled receptor to the 115,000 dalton receptor fragment. It is important to establish with certainty whether the fragments of phosphorylated receptor released by proteolysis retain phosphate following their formation. If so, phosphorylated fragments of receptor are attractive candidates for carriers of second messenger activity in EGF action [6].

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