Association of the Epidermal Growth Factor Receptor Kinase with the Detergent-insoluble Cytoskeleton of A431 Cells

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ABSTRACT The epidermal growth factor receptor (EGF-R) on human epidermoid carcinoma cells, A431, was found to be predominantly associated with the detergent-insoluble cytoskeleton, where it retained both a functional ligand-binding domain and an intrinsic tyrosine kinase activity. The EGF-R was constitutively associated with the A431 cytoskeleton; this association was not a consequence of adventitious binding. The EGF-R was associated with cytoskeletal elements both at the cell surface, within intracellular vesicles mediating the internalization of the hormone–receptor complex, and within lysosomes. The EGF-R became more stably associated with cytoskeletal elements after its internalization. The cytoskeletal association of the EGF-R was partially disrupted on suspension of adherent cells, indicating that alteration of cellular morphology influences the structural association of the EGF-R, and that the EGF-R is not intrinsically insoluble.

Cytoskeletons prepared from EGF-treated A431 cells, when incubated with γ-^{32}P-ATP, demonstrated enhanced autophosphorylation of the EGF-R in situ as well as the phosphorylation of several high molecular weight proteins. In this system, phosphorylation occurs between immobilized kinase and substrate. The EGF-R and several high molecular weight cytoskeletal proteins were phosphorylated on tyrosine residues; two of the latter proteins were phosphorylated transiently as a consequence of EGF action, suggesting that EGF caused the active redistribution of the protein substrates relative to protein kinases. The ability of EGF to stimulate protein phosphorylation in situ required treatment of intact cells at physiological temperatures; addition of EGF directly to cytoskeletons had no effect. These data suggest that the structural association of the EGF-R may play a role in cellular processing of the hormone, as well as in regulation of the EGF-R kinase activity and in specifying its cellular substrates.

Epidermal growth factor (EGF),[1] a potent mitogen, acts on its target cells by first binding to cell surface receptors, initiating a variety of changes (1) including rapid alterations in cell surface morphology (2, 3), cytoskeletal reorganization (4), and redistribution of the EGF receptors before internalization and degradation of the hormone (5–8). The mechanisms by which these changes occur, as well as those involved in the stimulation of DNA synthesis, are unknown. The discovery by Cohen and co-workers that the EGF receptor (EGF-R) possesses an intrinsic tyrosine kinase activity has focused attention on the role of protein phosphorylation as a critical event in regulating the biological activity of this hormone (9–

11). The importance of tyrosine phosphorylation, and particularly the autophosphorylation of the EGF-R, in regulating cellular proliferation is not entirely clear, since the stimulation of DNA synthesis is reported to be dissociated from EGF-stimulated tyrosine kinase activity (12, 13). Nonetheless, the similarity between the EGF-R kinase and retroviral gene products (14–16) has stimulated the search for the cellular mechanisms of hormone action.

The EGF-R is an integral membrane protein having sequence homology with the transforming gene product of the avian erythroblastosis virus erb-B (17). The EGF-R has a hormone binding domain exposed on the cell surface, while the protein kinase domain is located on the cytoplasmic face of the membrane. The cellular substrates for the EGF-R kinase have been elusive; aside from the autophosphorylated EGF-R, only a few proteins are phosphorylated on tyrosine

residues after exposure of A431 cells to EGF (15, 18). Our approach to investigating the role of protein phosphorylation in the mechanism of action of EGF was based on the hypothesis that hormone action is likely to produce stable changes in protein kinase activity and distribution relative to protein substrates. We have used a cell-free system which preserves cytoskeletal structures. In this system positional relationships between structurally associated constituents of the cell are retained, with protein phosphorylation in situ occurring between an immobilized enzyme and its protein substrates, reflecting close spatial relationships between the two (19).

Our understanding of the mechanisms governing the organization of proteins within biological membranes is currently undergoing rapid evolution. It is clear that the distribution in the membrane of a variety of membrane proteins is regulated in part through association with structural elements of the cell (20–22). It has subsequently been demonstrated that for a variety of cells, plasma membrane proteins are linked to a surface lamina or structural matrix, which persists upon removal of membrane lipids with nonionic detergents (23, 24). As a consequence, increasing attention has been directed at understanding membrane-cytoskeleton interactions and their role in regulating cellular architecture and metabolism (20, 25).

Of particular interest is the involvement of cytoskeletal elements in mediating the aggregation and internalization of cell surface receptors (26–28), and their participation in eliciting the biological effects of ligands. The cell surface receptors for a variety of ligands have been demonstrated to be associated with the Triton-insoluble cytoskeleton (29), some of which become associated as a consequence of ligand binding, e.g., membrane immunoglobulin (30), nerve growth factor (31), and chemotactic peptide (32). Cell surface receptors for peptide hormones and other ligands are mobile within the membrane; however, on ligand binding the receptors become progressively immobilized, concentrating over specialized regions of the plasma membrane where they are then internalized, frequently within coated vesicles (33–37). The internalized ligand–receptor complex is then translocated through the cytoplasm to associate with elements of the Golgi apparatus and with lysosomes (38–40). What role cytoskeletal structures play in mediating these events is presently unclear.

The present studies were initiated after the discovery of a 170-kD phosphoprotein present in a cytoskeletal preparation of A431 cells after in situ phosphorylation. This protein has been identified as the EGF-R; however, its presence on the detergent-insoluble cytoskeleton was surprising, since we had supposed this protein to be lost upon detergent solubilization of cellular membranes as reported by Cooper and Hunter (41). We report here that the EGF-R on A431 cells remains associated with the detergent-insoluble cytoskeleton both at the cell surface and at intracellular loci, and that the structural association of the receptor may modulate the activity and substrate specificity of the receptor-kinase.

MATERIALS AND METHODS

Materials: Epidermal growth factor was purchased from Collaborative Research (Lexington, MA). Radiolabeled ATP was prepared using Gamma Prep A from Promega Biotech (Madison, WI) and $^{32}P_i$ from ICN Radiochemicals (Irvine, CA). Cellulose thin layer plates were from Brinkmann Instruments Co. (Westbury, NY). Triton X-100 was from Sigma Chemical Co. (St. Louis, MO). Nonidet P-40 was from Polysciences, Inc. (Warrington, PA).

Cell Culture: Human epidermoid carcinoma cells, A431, were ob-

tained from Dr. M. A. Bothwell. The cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum.

Preparation of A431 Cytoskeletons: Adherent cells were grown in 16-mm wells, washed twice with phosphate-buffered saline (PBS), and 0.5 ml of the detergent-containing buffer was added for 2–3 min at 4°C; the resulting cytoskeletons were then washed briefly in detergent-free buffer. Three buffers were used. Buffer A contained 25 mM HEPES pH 7.4, 2 mM MnCl₂, 1 mM phenylmethylsulfonyl fluoride, and 0.15% or 0.5% Triton X-100. Buffer B was a modification of that of Burr et al. (19) containing 1% Triton, 25 mM HEPES pH 7.4, 0.3 M sucrose, 100 mM KCl, 2.5 mM MgCl₂, 2 mM MnCl₂, 1 mM CaCl₂, and 1 mM phenylmethylsulfonyl fluoride. Buffer C was that of Cooper and Hunter (41) and contained 0.5% Nonidet P-40, 10 mM PIPES pH 6.8, 100 mM KCl, 300 mM sucrose, 3 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride.

Phosphorylation of A431 Cytoskeletal Proteins: Subconfluent A431 cells were harvested from tissue culture plates and resuspended at a concentration of 0.5 × 10⁶ cells/ml in calcium- and magnesium-free PBS containing 1 mg/ml each of glucose and bovine serum albumin. EGF was added to the cell suspension at room temperature and at the appropriate times the cells were centrifuged 30 s in a Beckman Microfuge B (Beckman Instruments, Inc., Palo Alto, CA). The cell pellet was gently resuspended in cold buffer A containing 0.15% Triton, and incubated 2 min at 4°C. The resulting cytoskeletons were pelleted and resuspended in detergent-free lysis buffer, followed by addition of γ-³²P-ATP (10 μM, 5.5 × 10⁶ dpm/pmol), and incubated for 10 min at 4°C. The phosphorylation reaction was stopped by adding electrophoresis sample buffer and heating at 100°C for 5 min.

Phosphoamino Acid Analysis: The phosphoamino acid content of individual ³²P-labeled proteins was determined by excision of the labeled bands, acid hydrolysis, and separation of the labeled phosphoamino acids by electrophoresis at pH 3.5 exactly as described by Cooper et al. (42).

Binding of ¹²⁵I-EGF to A431 Cells: EGF was iodinated by the lactoperoxidase method (43). A431 cells were grown to near confluence in 16-mm wells. The cells were washed twice with PBS followed by addition of 0.5 ml of PBS containing ¹²⁵I-EGF. At the appropriate times, the ¹²⁵I-EGF-containing medium was removed and the cells washed three times with 1 ml of cold PBS, followed by addition of 1 ml of 1 N NaOH to solubilize the cellular protein (43). Nonspecific binding was measured in parallel incubations in the presence of a 200-fold or greater molar excess of unlabeled EGF, and was routinely <10% of total binding.

Cytoskeletally associated ¹²⁵I-EGF was measured as above except that the second and third washes were for 30 s each at 4°C in either buffer A or buffer B instead of PBS. This shorter procedure was used since Triton accelerates the dissociation of ¹²⁵I-EGF from its receptors (unpublished observations). The cell surface–bound hormone was measured by the method of Haigler et al. (44). The cells were washed once in PBS, incubated with 0.2 M acetic acid, 0.5 M NaCl for 2–5 min at 4°C, and washed briefly once more in the same buffer. The washes were combined, and acid-soluble and cell-bound radioactivity determined. Over 90% of cell surface–associated ¹²⁵I-EGF is selectively removed by this procedure (44).

Cross-linking of ¹²⁵I-EGF to the EGF Receptor: Radiolabeled EGF was covalently coupled to the EGF-R by the method of Comens et al. (45), using EGF iodinated by the Chloramine T method. A431 cells were grown in 16-mm wells and incubated for 30 min with 100 ng/ml of ¹²⁵I-EGF at 37°C. The cells were washed three times with cold PBS, 0.2 ml of cold buffer A containing 0.15% Triton was then added, and the cells incubated for an additional 2 min at 4°C. The buffer was removed and the cytoskeletons solubilized in electrophoresis sample buffer. Nonspecific binding was evaluated by a parallel incubation including a large molar excess of unlabeled EGF. 1% or less of the bound ¹²⁵I-EGF became covalently cross-linked to the EGF-R. The amount of ¹²⁵I-EGF-R in the various fractions was quantitated by excision of the labeled bands from the gels and determination of radioactivity in an LKB minigamma counter (LKB Instruments, Inc., Gaithersburg, MD).

Preparation of A431 Membranes and Vesicles: Membrane vesicles were prepared from A431 cells by the method of Cohen et al. (46). Plasma membranes were prepared by the method of Thom et al. (47).

Polyacrylamide Gel Electrophoresis: Cellular proteins were separated on 4–13% polyacrylamide gels by electrophoresis as described by Laemmli (48). The gels were stained with 0.1% Coomassie Blue, destained, dried, and exposed to Kodak XAR film with or without DuPont Lightning Plus intensifying screens (DuPont Co., Wilmington, DE).

Phospholipid Labeling: A431 cells were incubated in phosphate-free Dulbecco's modified Eagle's medium containing 50 μCi/ml ³²Pᵢ for 3 h at 37°C. The cells were washed once with PBS and extracted with buffer A containing 0.15 or 0.5% Triton, or with buffer C containing 0.5% Nonidet P-40, for 2 min at 4°C. The cytoskeletons were pelleted and the phospholipids

extracted three times with 1 ml of chloroform/methanol (2:1) for 1 min. The chloroform/methanol extracts were then combined with 0.6 ml water, and twice with 0.6 ml of chloroform/methanol/water (3:48:47). The lower phase was collected and ^{32}P measured by scintillation counting.

RESULTS

The cytoskeleton is operationally defined as those elements of the cell that are insoluble in nonionic detergent, including the surface lamina, the nucleus, fibrous structural elements, and associated proteins (23, 25).

The EGF-R was identified as a component of the detergent-insoluble cytoskeleton by covalent coupling of ^{125}I-EGF to 160- and 170-kD proteins (Fig. 1). The 170-kD protein is the native EGF-R, a fraction of which is proteolytically degraded to the lower molecular weight species (49). Lysis and extraction of the adherent A431 cells was done under a variety of conditions for 3 min at 4°C, to determine if by increasing detergent concentrations and ionic strength the EGF-R could be solubilized (Figs. 1 and 2).

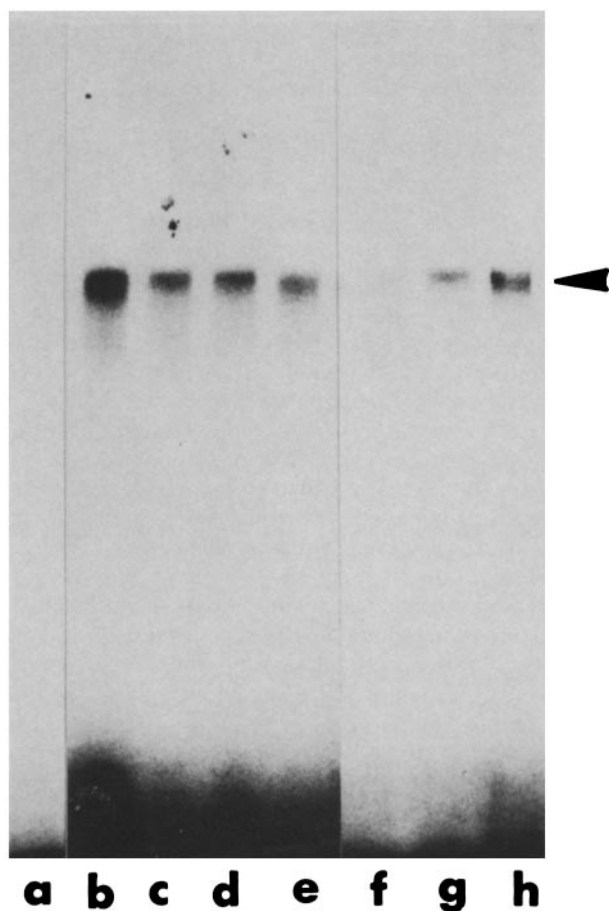Autoradiograms revealed that the EGF-R was predomi-

FIGURE 1 Association of cross-linked ^{125}I-EGF-receptor complexes with the detergent-insoluble cytoskeleton of A431 cells. Adherent A431 cells were incubated with 100 ng/ml of ^{125}I-EGF for 30 min at 37°C. The cells were extracted in buffer A containing 0.15% Triton (c and f), buffer A containing 0.5% Triton (d and g), or buffer C containing 0.5% Nonidet P-40 (e and h) for 3 min at 4°C. The detergent-insoluble (c–e) and soluble (f–h) fractions were collected and the proteins separated on 4–13% polyacrylamide gels. An autoradiogram of the ^{125}I-EGF-receptor complexes is shown (arrowhead). Nonspecific binding of unextracted cells incubated in the presence of a 100-fold excess of unlabeled EGF (a). Unextracted cells (b).
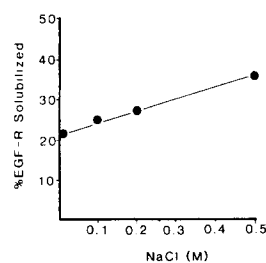

FIGURE 2 Effect of ionic strength on the cytoskeletal association of the EGF-R. Adherent A431 cells were incubated with 100 ng/ml of ^{125}I-EGF for 30 min at 37°C. The cells were then extracted for 3 min at 4°C in buffer A containing 0.5% Triton and the indicated concentrations of NaCl. Detergent soluble and insoluble fractions were collected and separated by gel electrophoresis. The labeled ^{125}I-EGF-receptor bands were excised and radioactivity determined.

TABLE I. Effect of A431 Membrane Vesicle Protein on Cytoskeletally Associated ^{125}I-EGF

	^{125}I-EGF	
	Total	0.15% Triton insoluble
	cpm	
Control	11,684	10,117
100 µg/ml Vesicle protein		9,520
200 µg/ml Vesicle protein		9,780

A431 cells were incubated with 2 ng/ml ^{125}I-EGF for 15 min at 37°C. The cells were washed three times with PBS for determination of total binding. Cytoskeletally associated ^{125}I-EGF was measured by first washing the cells once with PBS followed by two washes at 4°C with buffer A containing 0.15% Triton X-100. Membrane vesicles prepared from A431 cells were lysed in buffer A containing 0.15% Triton X-100 and adjusted to 100 or 200 µg of vesicle protein per milliliter. The cells were lysed at 4°C for 1 min with 0.5 ml of this solution containing vesicle protein, washed once with buffer A, and cytoskeletally associated radioactivity was determined. The EGF-R content of the vesicles was approximately 5- and 10-fold greater, respectively, than the amount of EGF-R present in the A431 cells in the assay.

nantly associated with the detergent-insoluble cytoskeleton, whether buffer A containing 0.15% or 0.5% Triton was used (Fig. 1). In a series of three experiments, extraction of A431 cells with 0.15 or 0.5% Triton solubilized from 12–17% and 20–35% of the EGF-R, respectively. Increasing the salt concentration to as much as 0.5 M resulted in solubilization of only an additional 15% of the EGF-R from the cytoskeleton (Fig. 2). Extraction in the buffer of Cooper and Hunter (buffer C) under these conditons solubilized ~50% of the EGF-R.

We compared the efficiency of lipid removal in the various buffer systems by labeling cellular phospholipids with ^{32}P. Triton in buffer A at concentrations of 0.15 and 0.5% removed 62 and 77%, respectively, of ^{32}P-labeled lipids. Buffer C, containing 0.5% Nonidet P-40, solubilized 89% of cellular phospholipids; this buffer was also more efficient in solubilizing cellular proteins, resulting in the loss of 42% of cellular protein, while buffer A containing 0.15% Triton solubilized 33%.

Cytoskeletal Association of the EGF-R Was Not Due to Adventitious Binding

A concern in these experiments was that the association of the EGF-R with the cytoskeleton may have been the result of adventitious binding of the solubilized EGF-R to cytoskeletal elements at the moment of cell lysis. We tested whether the EGF-R became nonspecifically associated with the cytoskeleton by lysing the cells in the presence of a large excess of plasma membrane protein which included the EGF-R (Table I). The presence of a large molar excess of EGF-R at the moment of cellular lysis had no effect on the amount of ^{125}I-
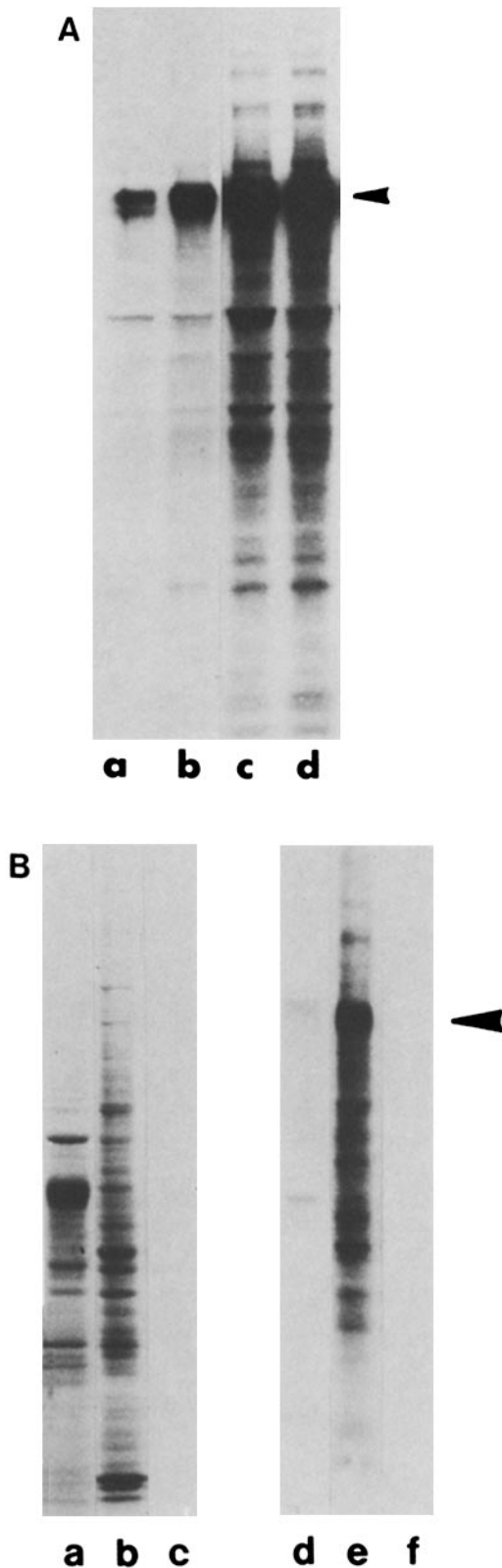
EGF associated with the cytoskeleton, suggesting that the EGF-R was not adventitiously bound to the cytoskeleton; rather, it was structurally associated at the time of cell lysis, and remained so even in the presence of an excess of soluble EGF-R. If the association of the EGF-R with the cytoskeleton was artifactual, then we would have expected a diminution of cytoskeletally associated ^{125}I-EGF, due to occupancy of non-specific cytoskeletal binding sites by exogenous EGF-R and other membrane proteins.

A second series of experiments was performed to test whether soluble ^{125}I-EGF-R would bind to A431 cytoskeletons. Radiolabeled EGF was cross-linked to EGF-R in purified membranes, and the uncross-linked ^{125}I-EGF was removed by addition of an excess of unlabeled EGF. The membranes were solubilized as described by Cohen (49) and then the solubilized ^{125}I-EGF-receptor complex collected by centrifugation. A431 cells were lysed in buffer A containing 0.15% Triton and 2,000, 5,000 or 10,000 cpm of soluble ^{125}I-EGF-receptor. Under these conditions, only ~3% (60, 152, and 255 cpm, respectively) of the soluble ^{125}I-EGF-R became associated with the A431 cytoskeletons. These data indicate that solubilized EGF-R does not bind to the cytoskeletons, and that the structural association of the EGF-R was not an artifact of the detergent-solubilization procedure.

Cytoskeletally Associated EGF-R Retains an EGF-stimulated Tyrosine Kinase Activity

The EGF-R that was retained with the detergent-insoluble cytoskeleton possessed an EGF-stimulated tyrosine-specific protein kinase activity. When A431 cells are treated with EGF in vivo, the EGF-R undergoes extensive EGF-stimulated autophosphorylation in situ (Fig. 3A). In addition, the phosphorylation of several high molecular weight (HMW) cytoskeletally associated proteins was stimulated as a consequence of EGF treatment. Stimulation of the cells with EGF results in a 50–100% increase in EGF-R autophosphorylation in situ; this value is somewhat less than that observed for isolated membranes or soluble EGF-R preparations (9, 46, 48). Maximal stimulation of EGF-R autophosphorylation occurred at EGF concentrations of 50 ng/ml.

The phosphorylation reaction occurs between immobilized protein kinase and protein substrates positioned in proximity to the active enzyme (19). Little or no protein kinase activity could be detected in solution during the phosphorylation reaction (Fig. 3B).

Binding of ^{125}I-EGF to A431 Cells and Cytoskeletons

The discovery that the EGF-R was associated with cytoskeletal elements led us to question whether the association

Figure 3   EGF-stimulated phosphorylation of A431 cytoskeletal proteins in situ. (A) A431 cells were incubated for 5 min at 22°C in the absence (a and c) or presence (b and d) of 50 ng/ml EGF. The cells were lysed in buffer A containing 0.15% Triton for 2 min at 4°C. The resulting cytoskeletons were pelleted and resuspended in buffer A containing 10 μM γ-^{32}P-ATP for 10 min at 4°C. The labeled proteins were separated on 4–13% polyacrylamide gels. An auto-radiogram of the labeled proteins is shown after an exposure of ~6

h (a and b) or 24 h (c and d). (B) A431 cells were incubated with 50 ng/ml EGF for 5 min at 22°C, lysed in buffer A containing 0.15% Triton, and the resulting cytoskeletons resuspended in detergent-free lysis buffer. Both the cellular lysate and cytoskeletons were incubated with γ-^{32}P-ATP for 10 min at 4°C. The cytoskeletons were then pelleted by centrifugation and the reaction supernatant recovered. The samples were separated on 4–13% polyacrylamide gels. Coomassie Blue-stained proteins (a–c) and the corresponding autoradiograms (d–f) of the cellular lysate (a and d), cytoskeletons (b and e), and reaction supernatant (c and f) are shown. The position of the EGF-R is indicated.

changed with cellular processing and internalization of the EGF-R complex, or under conditions where the EGF-R is mobile within the plane of the plasma membrane.

After binding of ^{125}I-EGF to A431 cells at 22°C, the ^{125}I-EGF remained predominantly associated with the cytoskeleton on extraction of the cells with 0.15% Triton or 1% Triton at higher ionic strength (Fig. 4). The latter buffer has previously been demonstrated to preserve cytoskeletal structure (except microtubules) (19).

If the binding was performed at 4°C, similar results were obtained. Although the total amount of ^{125}I-EGF bound at this temperature was lower than that at 37°C, most of the specifically bound ^{125}I-EGF was associated with the cytoskeleton. At low temperature, EGF-R complexes remain mobile as measured by the lateral diffusion coefficient, yet the concentration of receptors over coated pits and internalization are arrested (5, 7, 37). These data indicated that although the EGF-R could translocate within the membrane, it was associated with cytoskeletal elements of the cell. Similar data were obtained using ^{125}I-EGF concentrations ranging from 1–100 ng/ml.

Whether the EGF-R was constitutively associated with the A431 cytoskeleton, or rather became associated with it as a consequence of receptor occupancy, was tested by preparing cytoskeletons from untreated A431 cells and measuring the ability of the cytoskeletal preparations to specifically bind ^{125}I-EGF (Fig. 5). Radiolabeled EGF was specifically bound to the A431 cytoskeleton, indicating that the EGF-R was constitutively associated with the detergent-insoluble cytoskeleton, where it retained a functional ligand-binding domain.

We have frequently been able to bind ^{125}I-EGF to cytoskeletons at levels comparable to those of intact cells; however, varying the time of the solubilization step, inclusion of phosphatase inhibitors or ATP in the lysis buffer or binding solution all affect the degree of receptor binding in the cytoskeletal preparation (unpublished observations). These effects are likely to be related to modulation of the phosphorylation state of the EGF-R. The phosphorylation state of the receptor
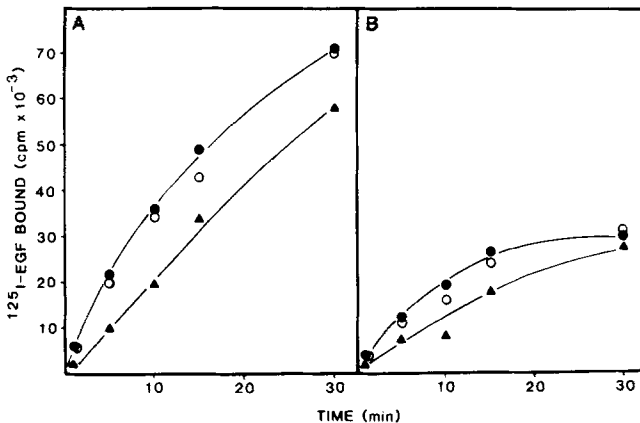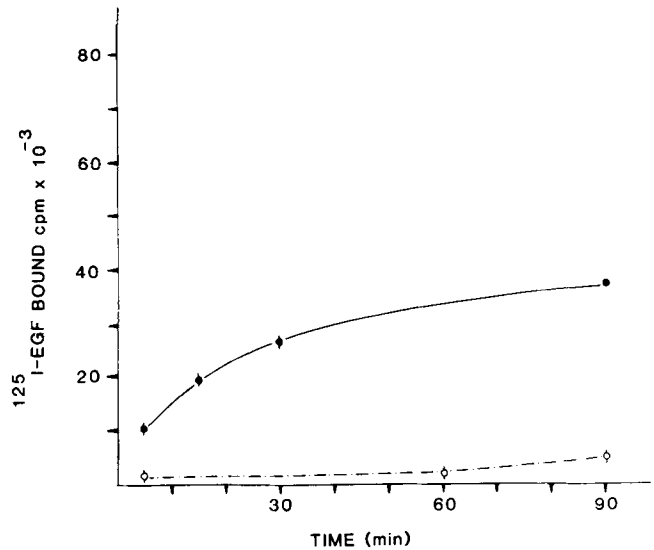

FIGURE 5 Binding of ^{125}I-EGF to A431 cytoskeletons. A431 cells grown in 16-mm wells were lysed by two washes at 4°C in buffer A containing 0.15% Triton, and then washed twice in detergent-free buffer A. The cytoskeletons were then incubated with 2 ng/ml ^{125}I-EGF for the indicated periods at 37°C, followed by two additional washes in cold buffer A (●). Nonspecific binding (O) was determined in a parallel incubation in the presence of a 500-fold molar excess of unlabeled EGF.

has a significant effect on receptor affinity and its ability to undergo ligand-stimulated autophosphorylation (50, 51).

The EGF-R Is Associated with the Cytoskeleton at Intracellular Loci

The observation that the EGF-R was associated with the cytoskeleton after several minutes at 37°C suggested that the EGF-R retained its association with the cytoskeleton after internalization. Haigler et al. (5) reported that on binding of EGF to A431 cells at 4°C, followed by removal of unbound ligand and incubation at 37°C, the EGF associated with the cell surface was rapidly internalized, appearing in cytoplasmic vesicles within 3 min. After 30 min at 37°C, ~80% of cell-associated EGF was located within lysosomes.

Using an experimental protocol similar to that of Haigler et al. (5), we found that EGF was rapidly internalized, as indicated by the rapid loss of acid-soluble radioactivity (Fig. 6). However, the ^{125}I-EGF–receptor complex remained associated with the Triton-insoluble cytoskeleton even at times when the EGF was predominantly located within lysosomes. These data demonstrate that the EGF-R was associated with cytoskeletal elements at the cell surface and at intracellular loci after its internalization.

The same experiment was repeated except that the ^{125}I-EGF was added at a concentration of 100 ng/ml, permitting the detection of ^{125}I-EGF cross-linked to the EGF-R (Fig. 7). The ^{125}I-EGF–receptor complex was nearly quantitatively associated with the cytoskeleton on extraction with 0.15% Triton or in 0.5% Nonidet P-40 in buffer C. Significantly, the degree of solubility of the EGF-R in buffer C under these conditions was substantially less than that shown in Fig. 1 (17% as compared with 50%), indicating that intracellular EGF-R was more stably associated with the cytoskeleton than was EGF-R on the cell surface. This change may be related to the recent report that the conformation of the EGF-R is

FIGURE 4 ^{125}I-EGF binding to A431 cells. A431 cells were incubated with ^{125}I-EGF (5 ng/ml) at 22°C (A) or 4°C (B). At the indicated times, the ^{125}I-EGF-containing medium was aspirated and the cells washed with PBS. The cells were then washed twice more with PBS (●), buffer A containing 0.15% Triton (O), or buffer B containing 1% Triton (▲), and the bound radioactivity determined. The points are the mean of duplicate determinations, which routinely differed by <5%. The data have been corrected for nonspecific binding, which was normally <10% of total binding.
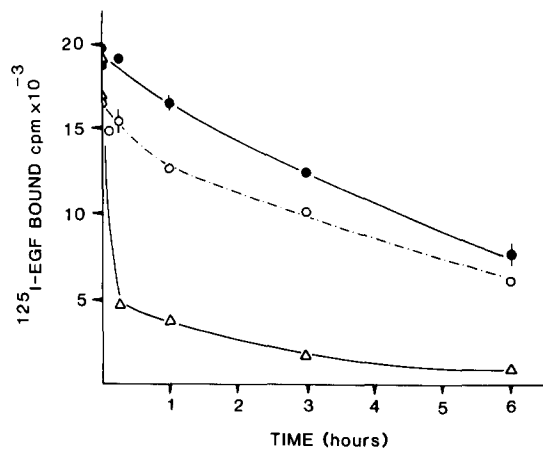
FIGURE 6 Redistribution of ^{125}I-EGF on A431 cells at physiological temperatures. A431 cells were incubated with 5 ng/ml ^{125}I-EGF for 40 min at 4°C, washed twice in PBS, and then warmed to 37°C. At the indicated times, the cells were washed once with PBS, then twice with PBS (O), buffer A containing 0.15% Triton (●), or with 0.2 M acetic acid, 0.5 M NaCl. The cell-associated and acid soluble (△) radioactivity was then measured. Nonspecific binding was <10% of total binding and has been subtracted.
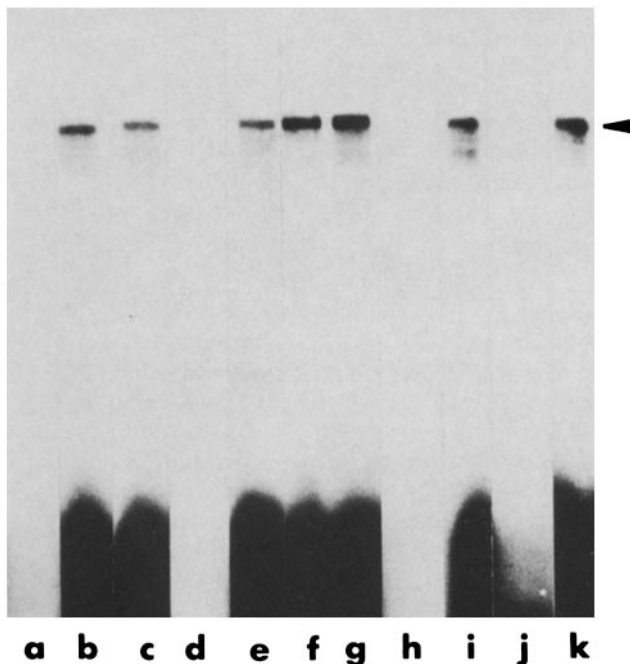

a b c d e f g h i j k

FIGURE 7 The EGF-R is cytoskeletally associated at intracellular loci. A431 cells were incubated with 100 ng/ml ^{125}I-EGF for 40 min at 4°C, and the cells washed twice to remove the unbound ^{125}I-EGF. The cells were warmed to 37°C and incubated at that temperature for 15 min. Whole cells were collected (b), or the cells extracted with two washes in buffer A containing 0.15% Triton and the detergent-insoluble cytoskeletal fraction (c) or detergent-soluble fraction (d) were collected. After 30 min of incubation at 37°C, unextracted cells (f), cytoskeletal (g), and soluble (h) fractions from cells extracted in buffer A with 0.15% Triton, or cytoskeletal (i) and soluble (j) fractions from cells extracted in buffer C, were collected. The ^{125}I-EGF-receptor complexes were determined to be internalized as indicated by trypsin resistance. After 15 min (e) or 30 min (k) at 37°C the cells were cooled to 4°C and incubated an additional 30 min with 0.5 mg/ml trypsin. Nonspecific binding was measured (a) in a parallel incubation in the presence of a 100-fold excess of unlabeled EGF followed by a 30-min incubation at 37°C. An autoradiogram of the ^{125}I-EGF-receptor complexes is shown.

altered upon its internalization in the endocytic vesicles (52). This experiment convincingly illustrates that very little solubilization of the EGF–receptor complex occurred upon detergent treatment of adherent A431 cells. The intracellular location of the ^{125}I-EGF-R was verified by incubating parallel samples at 4°C in the presence of 0.5 mg/ml trypsin. The ^{125}I-EGF–receptor complex was resistant to the proteinase. We concluded from these experiments that the EGF-R complex was cytoskeletally associated at intracellular loci, both within the cytoplasmic vesicles mediating hormone internalization and within lysosomes.

To verify that the cytoskeletally associated EGF-R was present within lysosomes, A431 cells were treated with the lysosomotropic agent chloroquine (Table II). Chloroquine inhibits the degradation of ^{125}I-EGF without affecting its internalization (53). Chloroquine-treated cells contained significantly greater amounts of ^{125}I-EGF, which was predominantly associated with the cytoskeleton, compared with untreated cells. These data demonstrate that EGF–receptor complexes remained associated with the cytoskeleton within intact lysosomes. The increase in amount of lysosomal EGF-R was not as great as that observed in fibroblasts (53), but is consistent with the observation that these cells do not down-regulate their receptors as efficiently (54, 55). It was possible that lysosomes were resistant to detergent at the concentrations used, resulting in the artifactual retention of labeled EGF within membrane-bounded compartments. This was shown to be unlikely as the lysis of the cells in 0.15 or 1% Triton resulted in the solubilization of >90% of lysosomal acid phosphatase (data not shown).

Solubilization of the EGF-R from A431 Cells in Suspension

We investigated whether other dramatic changes in cellular morphology might affect the structural association of the EGF-R. A431 cells were brought into suspension by treatment with 5 mM EDTA in calcium- and magnesium-free PBS. The resuspended cells were rounded, in contrast to the flattened and tightly adherent cells used in previous experiments. The EGF-R from cells in suspension, unlike adherent cells, were partially (30–50% of total EGF-R) solubilized upon treatment with 0.15% Triton (Fig. 8).

Two important conclusions can be drawn from this experiment. The data demonstrated that by removing the flat,

TABLE II. Effect of Chloroquine on ^{125}I-EGF Binding to A431 Cells

| | ^{125}I-EGF (cpm) | | |
	Total	Cytoskeletally associated	Cell surface
Untreated	12,675	10,200	1,800
0.1 mM Chloroquine	16,490	13,230	1,610

A431 cells were preincubated for 30 min at 37°C with 0.1 mM chloroquine, followed by incubation for 40 min at 4°C with 5 ng/ml ^{125}I-EGF. The radiolabeled EGF was removed, and the cells were washed twice with PBS and incubated for 3 h at 37°C in the presence or absence of 0.1 mM chloroquine, and washed with PBS. Total ^{125}I-EGF binding was determined after two further washes with PBS. Cytoskeletal binding was measured after two additional washes at 4°C in buffer A containing 0.15% Triton. Cell surface-associated EGF was measured by treating the cells with 0.2 M acetic acid and 0.5 M NaCl for 2 min at 4°C, removing all cell surface-associated EGF. Nonspecific binding was measured in a parallel incubation in the presence of 1 μg/ml unlabeled EGF and has been subtracted from the data.
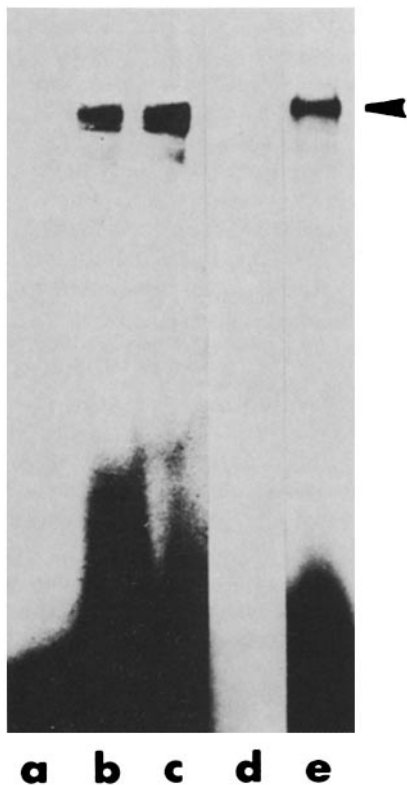
FIGURE 8 Solubilization of the EGF-R from A431 cells in suspension. A431 cells were left adherent to tissue culture wells (d and e), or brought into suspension (a–c) by incubation with 5 mM EDTA in calcium- and magnesium-free PBS and resuspension in the same buffer without EDTA. ^{125}I-EGF (50 ng/ml) was added and the cells incubated for 30 min at 37°C in the presence (a) or absence (b and d) of 1 μg/ml unlabeled EGF. The cells were pelleted, resuspended in buffer A containing 0.15% Triton, and incubated for 2 min at 4°C. Cytoskeletal and soluble fractions were recovered after centrifugation and separated by gel electrophoresis. An autoradiogram of the ^{125}I-EGF-receptor complexes is shown and the position of the EGF-R indicated.

adherent cells from their substratum, the association of the EGF-R with the cytoskeleton was partially disrupted as a consequence of the altered cytoskeletal organization in the unattached, rounded cells. Further, the observation that under some conditions the EGF-R can become detergent-soluble provides additional evidence that the association of the EGF-R with the cytoskeleton is neither the result of artifactual binding due to detergent treatment, nor due to an inherent insolubility of the receptor. We have been unable to demonstrate that calcium plays a significant role, since the addition of either calcium or EGTA to the lysis buffer had no effect on the association of the EGF-R with the cytoskeleton in adherent cells (data not shown).

Stimulation of the Cytoskeletally Associated EGF–Receptor Kinase Requires Treatment of Intact Cells at Physiological Temperatures

The ability of EGF to stimulate the autophosphorylation of the EGF-R present on cytoskeletons prepared from untreated A431 cells was tested. Addition of EGF (100–200 ng/ml) directly to A431 cytoskeletons failed in all cases (six experiments) to stimulate the incorporation of ^{32}P into the EGF-R when incubated either at 4° or 22°C for 5–15 min

(Roy, L., S. Alam, and G. Landreth, manuscript in preparation).

Whereas EGF treatment of A431 cells at 22°C stimulated the autophosphorylation of the EGF-R by 50–100% if the reaction was carried out at 4°C, little or no (0–20%) stimulation of EGF-R autophosphorylation was observed (Roy, L., S. Alam, and G. Landreth, manuscript in preparation). These data indicate that the activation of the cytoskeletally associated EGF-R requires treatment of intact A431 cells at physiological temperatures, suggesting that ligand occupancy alone is not sufficient to activate the structurally associated receptor-kinase in situ.

Time Course of EGF-stimulated Phosphorylation of Cytoskeletal Proteins

A431 cells were treated with EGF (50 ng/ml) at 22°C for 30 s to 20 min, cytoskeletons prepared, and then incubated for 10 min with γ-^{32}P-ATP. EGF stimulated the phosphorylation of several HMW cytoskeletal proteins in addition to the autophosphorylation of the EGF-R (Fig. 9). The phosphorylation in situ of two of these proteins had distinctly different
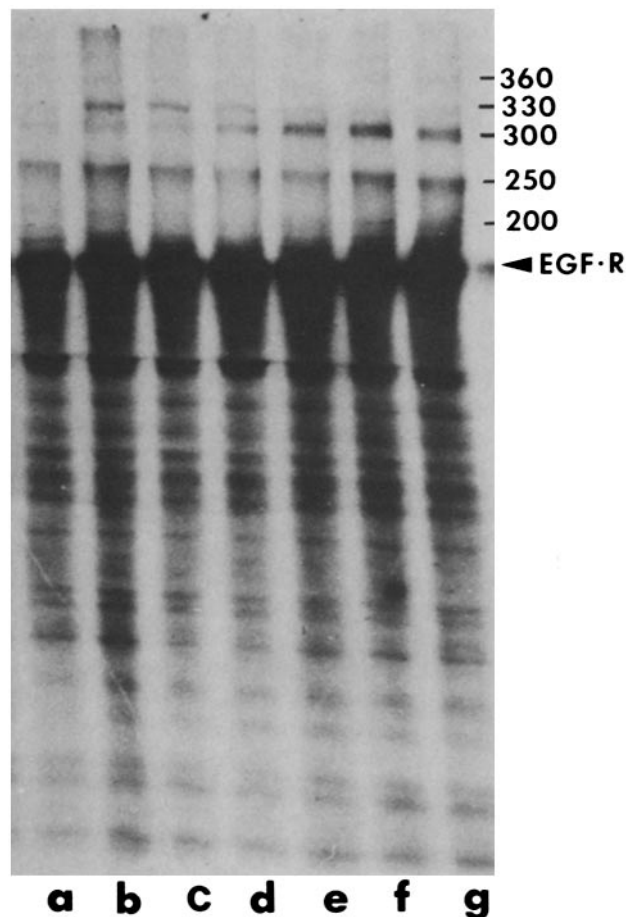

FIGURE 9 Time course of EGF-stimulated phosphorylation of cytoskeletal proteins in situ. A431 cells were incubated with EGF (50 ng/ml) for 30 s–20 min at 22°C, cytoskeletons prepared using buffer A with 0.15% Triton, and then incubated with γ-[^{32}P]ATP for 10 min at 4°C. Autoradiograms of the labeled cytoskeletal proteins are shown from untreated cells (a); and from cells exposed to EGF for 30 s (b), and 1 (c), 2 (d), 5 (e), 10 (f), and 20 (g) min. The approximate molecular weights of the responsive proteins were 330 and 300 kD.

time courses. The phosphorylation of a 330-kD protein is transient, with enhanced labeling occurring within 30 s of hormone exposure and disappearing after 5 min with EGF. The phosphorylation of a 300-kD protein is much different; this protein becomes labeled within 5 min of EGF exposure and does not diminish until after 20 min with EGF. The time dependence of ^{32}P-labeling of the EGF-R demonstrates that the EGF-R kinase activity is persistent throughout the entire period, with little difference in receptor autophosphorylation between short and long exposures to EGF. These data demonstrate that not only are other cytoskeletal proteins substrates for EGF-stimulated protein kinase activity, but the availability of the protein substrates to the hormonally stimulated kinase changes rapidly as a consequence of hormone action.

Phosphoamino Acid Analysis of Cytoskeletal Phosphoproteins

The HMW cytoskeletal proteins whose phosphorylation in situ was enhanced by EGF treatment of A431 cells were excised from the gel, hydrolyzed, and their phosphoamino acid composition determined. The HMW cytoskeletal proteins possessed phosphotyrosine as well as phosphoserine and phosphothreonine residues (Fig. 10; Table III). The amount of phosphotyrosine in these proteins varied between 24–50% of total protein phosphate. The relative rarity of tyrosine kinases other than the EGF-R in these cells indicates the involvement of the EGF-R in the phosphorylation of these proteins. The presence of phosphoserine residues suggests that these proteins are also substrates for other cytoskeletally associated protein kinases. The EGF-R appears to be labeled
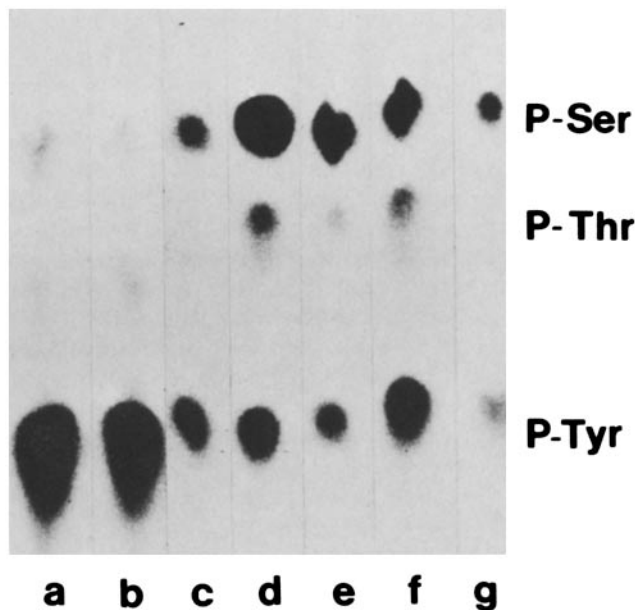

FIGURE 10 Phosphoamino acid analysis of A431 cytoskeletal proteins. The radiolabeled cytoskeletal proteins from untreated A431 cells (a) or cells treated for 5 min at 22°C with 50 ng/ml EGF were phosphorylated in situ and separated on 5–15% polyacrylamide gels. Those proteins whose phosphorylation was stimulated by EGF treatment are shown in Fig. 9; these bands were excised, hydrolyzed, and the phosphoamino acids separated by electrophoresis on a thin-layer plate at pH 3.5; (a) EGF-R, basal; (b) EGF-R, EGF-stimulated; (c) 200-, (d) 250-, (e) 300-, (f) 330-, and (g) 360-kD cytoskeletal proteins. The positions of phosphoamino acid standards are shown.

TABLE III. *Phosphoamino Acid Composition of A431 Cytoskeletal Proteins*

Protein	P-Ser	P-Thr	P-Tyr
kD	%	%	%
360	53	19	28
330	37	23	40
300	42	35	23
250	57	19	24
200	34	16	50
EGF-R (basal)	12	5	83
EGF-R (stimulated)	9	8	83

A431 cells were incubated in the absence or presence of 100 ng/ml of EGF for 5 min at 22°C, cytoskeletons prepared and incubated with 10 μM γ-^{32}P-ATP for 15 min at 4°C. The cytoskeletal proteins were separated on 4–13% SDS polyacrylamide gels, stained, dried, and autoradiograms obtained. Cytoskeletal proteins whose phosphorylation was stimulated by EGF are indicated in Fig. 9; these proteins were excised from the gel and hydrolyzed, and phosphoamino acids were separated. The labeled phosphoamino acids were scraped from the thin layer plates, and radioactivity was determined. The data are expressed as the percentage of radioactivity associated with each amino acid.

almost exclusively on tyrosine residues, which is consistent with other observations on the in vitro phosphorylation of this protein (11); however, we detected small amounts of labeled phosphoserine and phosphothreonine residues, suggesting that the EGF-R may also be a substrate for other protein kinases in situ. We have not ruled out that other phosphoproteins may have co-migrated with the EGF-R, which might account for the presence of phosphoserine and phosphothreonine residues.

DISCUSSION

We report here that the EGF-R on A431 cells is constitutively associated with the detergent-insoluble cytoskeleton, where it retains an active ligand-binding activity and tyrosine-specific protein kinase activity.

The present studies were initiated in an attempt to determine whether EGF produced stable alterations in A431 cells which persisted in the detergent-insoluble cytoskeleton, resulting in changes in phosphorylation of cytoskeletally associated proteins in situ, analogous to those observed on hormone treatment of PC12 cells (56). We were surprised to find that the major phosphorylated species present in the cytoskeletons was the EGF-R, since we had anticipated that the detergent treatment would result in the loss of this integral membrane protein, as reported by Cooper and Hunter (41). It is possible that in the experiments of Cooper and Hunter, where the EGF-R was detected by immunoprecipitation, that the EGF-R in particulate fractions was not detected due to incomplete solubilization of the receptor from the cytoskeleton (41).

The relative insolubility of the EGF-R was noted by Linsley and Fox (57), who reported that on treatment of A431 membranes with Triton at concentrations up to 10%, ~50% of the EGF-R was sedimented after low-speed centrifugation; moreover, treatment of the membranes with sodium dodecyl sulfate, urea, or high salt concentrations failed to completely solubilize the EGF-R. The EGF-R, once solubilized, has a heterogeneous size distribution, eluting on gel filtration at positions well ahead of that expected of the 170-kD EGF-R (49, 57).

The persistent association of the EGF-R with the detergent-insoluble cytoskeleton suggests that the EGF-R interacts with components of the surface lamina, rendering it insoluble. The

data argue that this interaction is a specific one of importance in the processing of the hormone-receptor complex. First, disorganization of cellular morphology on suspension of the cells results in the solubilization of ~30–50% of the EGF-R. This is likely to be related to the twofold reduction in the rate of EGF internalization and degradation by the suspended cells compared with adherent ones (7). Second, the interaction of the EGF-R with cytoskeletal elements is stabilized after internalization of the hormone-receptor complex as indicated by the decrease in EGF-R solubility in buffer C after internalization (Figs. 1 and 7). We interpret these data as evidence that the EGF-R at the cell surface interacts with components of the surface lamina, even under conditions where the receptor has been demonstrated to be mobile. These observations suggest that the EGF-R may interact with structural elements, perhaps transiently. These types of interactions may account for the diminished diffusion coefficients of proteins in biological membranes compared with pure phospholipid bilayers (7, 36, 37, 58, 59). These structural interactions of the EGF-R may also be involved in the vectorial movement of occupied receptors to internalization sites and then directing the movement between intracellular organelles. One striking finding in this study was that the EGF-R retained its cytoskeletal association after internalization and translocation to various intracellular loci including lysosomes. We had supposed that these intracellular vesicles and their contents would be lost on detergent treatment. These observations suggest that the protein matrix constituting the surface lamina may be retained as a component of the endocytotic vesicle, and may indicate that intracellular membranous systems also possess such a structural matrix as a constituent of their membranes. It is not unreasonable that a structural matrix and associated proteins may specify not only characteristic morphology of intracellular membranous compartments, but also may play a central role in establishing the biochemical individuality of these membranes (60, 61).

The association of the EGF-R with the cytoskeleton is not likely to involve cellular microtubules or microfilaments since treatment of A431 cells with either 50 μM colchicine or 10 μM cytochalasin D has no effect on the association of the EGF-R with the cytoskeleton. Furthermore, incubation of ^{125}I-EGF-labeled cytoskeletons with DNase I was without effect (data not shown).

We have investigated EGF-stimulated protein phosphorylation using a cell-free system in which structural relationships are preserved (19, 62). The in situ phosphorylation observed in this preparation reflects the activity of a structurally associated enzyme acting on protein substrates that are also immobilized, and implies that close spatial relationships exist between the two species. The ability to detect EGF-stimulated phosphorylation of cytoskeletal proteins is likely to be due in part to the loss of active phosphoprotein phosphatases on removal of cellular membranes and soluble proteins. The use of this system has allowed us to discern subtle rearrangements of proteins relative to the respective kinases (or vice versa), and had validated this approach to investigation of hormone action, since we have demonstrated that EGF does indeed produce stable changes that persist in the detergent-insoluble cytoskeleton. The evanescent phosphorylation of the HMW cytoskeletal proteins suggests that on ligand binding and activation of the EGF-R kinase, structural relationships are changed such that the HMW proteins become accessible to the kinase as a transient consequence of hormone action. The

tyrosine kinase activity in these cells is due almost entirely to the EGF-R as a result of amplification of the EGF-R gene (63).

We have chosen to use A431 cells in suspension for phosphorylation studies principally because we could reproducibly demonstrate EGF-stimulated protein phosphorylation in this preparation. Similar studies with adherent cells have given inconsistent results, due mainly to very high basal levels of EGF-R autophosphorylation in situ.

The fidelity with which this system reflects the situation in vivo is unclear, given the relaxed specificity of protein phosphorylation in vitro (42). The advantage of this preparation is that it allows identification of putative protein substrates of the EGF-R, since these proteins lie in proximity to the kinase. Indeed, using this rationale, the HMW cytoskeletal proteins have been shown to be phosphorylated in vivo, and we are currently attempting to identify the labeled residues.

The HMW cytoskeletal proteins were also substrates for other kinases, resulting in the in situ phosphorylation at serine and threonine residues. Whether EGF stimulates the activity of a separate class of kinases in situ is unclear. However, in vivo protein phosphorylation on these residues is enhanced by EGF treatment, including phosphorylation of the EGF-R (15, 50).

The inability of A431 cells treated with EGF at low temperature to undergo EGF-stimulated receptor autophosphorylation in situ was an unexpected observation, and suggests that cellular events occurring at elevated temperatures are required for activation of the EGF-R kinase. These data are reminiscent of the earlier observations of Cohen et al. who reported that activation of the EGF-R kinase in A431 membranes required incubation at 22°C and did not occur at 4°C (9). We also found that EGF treatment of cytoskeletons prepared from untreated A431 cells did not activate EGF-R autophosphorylation in situ at either 4° or 37°C. This also is surprising, given the extensive data on the in vitro phosphorylation of this protein. The significance of these findings is that the structural association of the EGF-R has a dramatic effect on the ability of EGF to stimulate the tyrosine kinase activity despite a functional binding domain. The structural association of the receptor may modulate both the activity of the enzyme and the availability of phosphorylation sites. In the latter case the immobilization of the EGF-R may hinder its access to phosphorylation sites on the EGF-R itself in intramolecular reactions. It is also possible that in untreated A431 cells the EGF-R is not in proximity to other EGF-R, which normally occurs as a consequence of ligand-stimulated receptor aggregation, permitting intermolecular reactions to occur.

The relevance of the tyrosine kinase activity of the EGF-R to the biological effects of EGF is currently uncertain (12, 13). It is of interest that an immediate effect of EGF on A431 cells is a rapid change in cell surface morphology, with the development of membrane ruffles and lamellipodia, subsiding within 10 min of EGF exposure (2, 3). These changes are remarkably similar to those observed in Rous sarcoma virus-infected cells (64, 65), the transforming gene product of which is also a tyrosine kinase. The latter enzyme is at least in part associated with cytoskeletal elements on the cytoplasmic face of the plasma membrane (19, 66).

The observation that the EGF-R is associated with structural elements of the cell suggests that these structural inter-

actions may play a role in the cellular dynamics of the EGF-R and may modulate its activity and substrate specificity.

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