

# Direct Visualization of the Phosphorylated Epidermal Growth Factor Receptor during Its Internalization in A-431 Cells

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**Abstract.** Epidermal growth factor (EGF) rapidly stimulates receptor autophosphorylation in A-431 cells. After 1 min the phosphorylated receptor can be identified at the plasma membrane using an antiphosphotyrosine antibody. With further incubation at 37°C, ~50% of the phosphorylated EGF receptor was internalized ( $t_{1/2} = 5$  min) and associated with the tubulovesicular system and later with multivesicular bodies, but not the nucleus. During this period, there was no change in the extent or sites of phosphorylation. At all times the phosphotyrosine remained on the cytoplas-

mic side of the membrane, opposite to the EGF ligand identified by anti-EGF antibody. These data indicate that (a) the tyrosine-phosphorylated EGF receptor is internalized in its activated form providing a mechanism for translocation of the receptor kinase to substrates in the cell interior; (b) the internalized receptor remains intact for at least 60 min, does not associate with the nucleus, and does not generate any tyrosine-phosphorylated fragments; and (c) tyrosine phosphorylation alone is not the signal for receptor internalization.

THE receptor for epidermal growth factor (EGF)<sup>1</sup> is a 170-kD integral membrane glycoprotein that undergoes autophosphorylation on tyrosine residues during EGF binding (Carpenter et al., 1979; Cohen et al., 1980; Ushiro and Cohen, 1980; Hunter and Cooper, 1981; Cohen et al., 1982; Buhrow et al., 1982; King and Cooper, 1986). It is composed of at least three functional domains: an extracellular amino-terminal region that binds EGF with high affinity (Ullrich et al., 1984); a single hydrophobic domain which serves as the transmembrane-spanning region (Ullrich et al., 1984); and an intracellular domain which contains an ATP-binding site, a tyrosine kinase catalytic site, and several sites of autophosphorylation (Russo et al., 1985; Basu et al., 1984; Ullrich et al., 1984; Downward et al., 1984; King and Cooper, 1986). In many cell types, the binding of EGF induces a broad range of early and delayed biologic responses eventually leading to mitogenesis. Activation of the tyrosine kinase activity (Bertics and Gill, 1985) and autophosphorylation are believed to represent the primary molecular events initiating the transmission of the mitogenic signal inside of the cell.

Possible mechanisms by which the activated EGF receptor initiates its actions could involve endocytosis of the EGF receptor complex to gain access to intracellular substrates

(Cohen and Fava, 1985) or proteolysis of the receptor to generate a fragment which acts as an intracellular mediator of EGF action (Das and Fox, 1978). Various approaches have been used to locate EGF receptors in the intact cell and follow their movement during EGF stimulation (Haigler et al., 1978, 1979; McKanna et al., 1979; Gorden et al., 1978; Schlessinger et al., 1978; Zidovetzki et al., 1981; Carpentier et al., 1981, 1986; Boonstra et al., 1985; Dunn et al., 1986; Miller et al., 1986; Kris et al., 1985). Cohen and Fava (1985) have demonstrated that the active EGF receptor kinase appears to be internalized in A-431 cells by isolation of membrane fractions by differential centrifugation. In this report, we have used polyclonal anti-phosphotyrosine antibody ( $\alpha$ PTyr) (Pang et al., 1985) and a combination of morphological and biochemical techniques to define precisely the movement and chemical nature of the EGF receptors that undergo tyrosine autophosphorylation during EGF binding to intact A-431 cells. Using both fluorescence microscopy and electron microscopy of protein A-gold particles, we find that the phosphotyrosine-containing EGF receptors are located entirely at the plasma membrane immediately after EGF binding, but after 10–30 min at 37°C, half of these receptors are internalized and associate with the tubulovesicular system and multivesicular bodies but not with the nucleus. During this period of movement, the amount of EGF receptor remains constant and the phosphorylation sites are unchanged. Thus, the activated, intact EGF receptor is internalized and moves through the cell where it can interact with various intracellular substrates.

1. *Abbreviations used in this paper:*  $\alpha$ EGF, anti-EGF antibody;  $\alpha$ EGFR, anti-EGF receptor antibody RK2;  $\alpha$ PTyr, anti-phosphotyrosine antibody; EGF, epidermal growth factor.

## Materials and Methods

### Materials

Human A-431 epidermoid carcinoma cells were obtained from G. Todaro (National Cancer Institute, Frederick, MD) or the American Type Tissue Culture Collection (Rockville, MD) and maintained in DME (Gibco, Grand Island, NY) with 10% (vol/vol) FCS (Gibco) in 5% CO<sub>2</sub>/95% air atmosphere at 37°C. For morphological analysis, the cells were grown in 35-mm dishes (Falcon Labware, Oxnard, CA) and used when they reached subconfluence. For phosphorylation studies, the cells were grown to confluence in 150-mm dishes. The  $\alpha$ PTyr was prepared in rabbits by injection of *N*-bromoacetyl-0-phosphotyramine conjugated to keyhole limpet hemocyanin as previously described (Pang et al., 1985a, b). The antibody was purified from the serum by affinity chromatography on phosphotyramine immobilized to Affi-gel (Bio-Rad Laboratories, Richmond, CA). The anti-EGF receptor antibody RK2 ( $\alpha$ EGFR) was obtained from a rabbit immunized against a synthetic peptide with the same amino acid sequence as a portion of the intracytoplasmic domain of the EGF receptor (Kris et al., 1985). It was kindly provided to us by Dr. Y. Schlessinger (Weizmann Institute of Science, Rehovot, Israel). The anti-EGF antibody ( $\alpha$ EGF) was obtained from Bethesda Research Laboratories (Bethesda, MD). Colloidal gold particles (7 nm) were coupled to protein A (*Staphylococcus aureus* protein A; Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (Frens, 1973; Orci et al., 1984). Receptor grade EGF was obtained from Collaborative Research (Waltham, MA). FITC-conjugated goat anti-rabbit IgG was obtained from Cappel Laboratories (Malvern, PA). [<sup>32</sup>P]Orthophosphate and Triton X-100 were from New England Nuclear (Boston, MA); phosphoamino acids, sodium fluoride, sodium vanadate, aprotinin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO). Pansorbin was from Calbiochem-Behring Corp. (San Diego, CA) and [<sup>125</sup>I]EGF (140  $\mu$ Ci/ $\mu$ g) was from Amersham (Buckinghamshire, England). The reagents for SDS-PAGE and the RP-318 reverse-phase HPLC column were purchased from Bio-Rad Laboratories; and acetonitrile was from Fischer & Porter Co., (Warminster, PA).

### Preparation of A-431 Cells for Morphological Analysis

12 h before each experiment, the A-431 cells were washed and incubated with serum-free DME. Immediately before the experiment, the cells were washed once with Earle's balanced salt solution (EBSS) containing 120 mM NaCl, 15 mM KCl, 1 mM MgSO<sub>4</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 30 mM NaHCO<sub>3</sub>, 5 mM glucose, and 20 mM Hepes (pH 7.4), and incubated for 10 min at 4 or 37°C in 2 ml of EBSS. The cells were incubated for various periods of time (0–120 min) in the presence or absence of 100 nM EGF at 4° or 37°C. For electron microscopic autoradiographic analysis [<sup>125</sup>I]EGF (0.1 nM) was used instead of unlabeled EGF. When indicated, EGF was replaced by insulin (100  $\mu$ M) or PMA (50 nM). After these incubations, the cells were washed twice at 4°C with EBSS and fixed either in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS (0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4) for 30 min at 22°C (immunocytochemical analysis) or in 2.5% glutaraldehyde in 0.1 M Na phosphate buffer pH 7.4 for 30 min at 22°C (autoradiographic analysis).

To identify the phosphotyrosine residues in whole cells, fixed A-431 cells were kept attached to tissue culture dishes in PBS (pH 7.4) and used for immunofluorescence. Alternatively, to localize phosphotyrosine residues both on semi-thin and thin sections, the cells were scraped from the dishes with a rubber policeman, embedded in Agar which was cut into small blocks (2–3 mm<sup>3</sup>), dehydrated with ethanol, and embedded in Epon 812 or processed for low-temperature embedding in Lowicryl K4M (Carlemalm et al., 1980; Roth et al., 1978). For autoradiographic analysis, fixed cells were dehydrated in the dishes and processed for EM autoradiography as previously described (Carpentier et al., 1978). Cells were cut parallel to the plane of the culture dish.

### Localization of Phosphotyrosine-containing Proteins by Light Microscopy

To localize PTyr residues in whole cells fixed but not embedded, A431 cells in petri dishes were permeabilized by a rapid dehydration in graded ethanol followed by a rapid rehydration. Cells were then washed with PBS (pH 7.4) and incubated for 2 h at 22°C in PBS containing  $\alpha$ PTyr (5–10  $\mu$ g/ml). After washing with PBS, the cells were incubated with FITC-conjugated goat anti-rabbit IgG at a dilution of 1:50 for 60 min at room temperature, rinsed in PBS, and counterstained with Evans blue before examination in a Leitz

Ortholux fluorescence microscope. Control experiments included incubation of nonpermeabilized cells. The specificity of  $\alpha$ PTyr was verified by testing the inhibitory potency of 2 mM phosphoserine, 2 mM phosphothreonine, or 2 mM phosphotyrosine on the fluorescent reactions produced by the antibody.

Semi-thin sections (0.5–1  $\mu$ m) of Epon-embedded A-431 cells were collected on glass slides and the Epon was removed according to the procedure of Maxwell (1978). After washings in distilled water, Epon-free sections were covered with a drop of  $\alpha$ PTyr (5–10  $\mu$ g/ml) for 2 h at room temperature in a moist chamber. After washing with PBS, the sections were incubated with FITC-conjugated goat anti-rabbit IgG for 60 min at room temperature as previously described (Orci et al., 1985), washed with PBS, contrasted with Evans blue, and examined by fluorescence microscopy.

### Localization of Phosphotyrosine-containing Proteins at the Electron Microscopic Level

Thin sections of Lowicryl-embedded A-431 cells were collected on nickel grids and immunolabeled by the protein A-gold method (Roth et al., 1978). Sections were incubated overnight at 4°C in a moist chamber by floating the grids on a drop of the  $\alpha$ PTyr. The grids were then rinsed with distilled water and incubated with the protein A-gold solution (dilution 1:70) for 1 h at room temperature with washing in distilled water between each incubation step. Immunolabeled sections were double stained with uranyl acetate and lead citrate before examination at the electron microscope.

To quantify these results, two separate experiments were performed. In each case, 50 pictures were randomly photographed. Randomness was insured by photographing the cell sections located nearest to the four corners of 12 successive grid holes. Pictures were taken at an initial magnification of 19,000. The number of gold particles per  $\mu$ m of plasma membrane and per  $\mu$ m<sup>2</sup> of cellular compartment was evaluated on negatives at a final magnification of 68,000. The intensity of labeling was assessed as the number of gold particles/ $\mu$ m<sup>2</sup> on the following compartments: lysosome-like structure (including multivesicular bodies and large vacuolar structures containing heterogenous material), tubulo-vesicular structures (small electron-lucent tubules and vesicles), mitochondria, nucleus, or as the number of gold particles/ $\mu$ m of plasma membrane.

### Localization of [<sup>125</sup>I]EGF by Quantitative EM Autoradiography

For each incubation time analyzed, three Epon blocks were prepared and three sections were cut from each block. Thus for each time point of incubation nine separate grids were examined out of which 200–300 autoradiographic grains were randomly photographed from all cells judged to be morphologically intact.

The association of the labeled material with the plasma membrane of the A-431 cells was assessed quantitatively by the method of Salpeter et al. (1977). The percentage of total number of grains was plotted as a function of the distance between the grain center and the closest plasma membrane as previously described (Carpentier et al., 1978).

The relationship of autoradiographic grains to intracellular structures was determined by superimposing each autoradiographic grain with a 250-nm-diam circle. Grains overlying the cytoplasm (>250 nm from the plasma membrane) were divided into the following classes based on their relation to the following structures: tubulo-vesicular structure, lysosomal structures (see above), mitochondria, and nucleus. The quantification was carried out as previously described (Carpentier et al., 1986).

### Immunoprecipitation of <sup>32</sup>P-labeled Proteins

Confluent A-431 cells were incubated for 2 h with 10 ml of phosphate-free and serum-free RPMI 1640 medium (Gibco) containing carrier-free [<sup>32</sup>P]phosphate (0.5 mCi/ml). EGF (100 nM) was added and the incubation of the cells was continued at 37°C for the indicated time intervals. The experiments were stopped by removing the incubation medium and rapidly freezing the cell monolayers with liquid nitrogen. The monolayers were thawed and solubilized immediately at 4°C with 2 ml of a solution containing 50 mM Hepes (pH 7.4), 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/ml aprotinin, and 2 mM PMSF. The cells were scraped from the dishes and the insoluble material was sedimented by centrifugation at 50,000 rpm in a 70.1 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) for 60 min and discarded.

Phosphotyrosine-containing proteins were purified from the soluble

extract by immunoprecipitation with 3  $\mu$ g of  $\alpha$ PTyr. The antibody was immobilized on Pansorbin and the precipitate was washed three times with a solution containing 50 mM Hepes, pH 7.4, 100 mM NaF, 2 mM  $\text{Na}_3\text{PO}_4$ , 1% Triton X-100, 0.1% SDS. The phosphoproteins were eluted from washed precipitates by incubation for 2 h at 22°C with 50 mM *p*-nitrophenylphosphate in 50 mM Hepes. The eluted proteins were reduced with 100 mM DTT and separated by SDS-PAGE on 10% resolving polyacrylamide gels. For some experiments the eluted proteins were reimmunoprecipitated with  $\alpha$ EGFR (from J. Schlessinger, The Weizmann Institute of Science, Rehovot, Israel) (Kris et al., 1985) and then separated on a 7.5% resolving gel as previously described (Kasuga et al., 1985). The phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-Omat film and an intensifying screen. The radioactivity in gel fragments was quantified by Cerenkov counting.

### Immunoblot Analysis

A-431 cells cultured and incubated with or without EGF as described above were solubilized as for the immunoprecipitation studies. SDS-PAGE was performed in 7.5% polyacrylamide by the method of Laemmli (1970). After PAGE, the gels were removed and placed in a multiple-layer "sandwich": one electrode on a Scotch Brite pad (Minnesota Mining and Manufacturing Co., St. Paul, MN), two 3 MM paper filters (Whatman Inc., Clifton, NJ), the polyacrylamide gel, a sheet of nitrocellulose paper, two more paper filters (Whatman Inc.), and another Scotch Brite pad (Minnesota Mining and Manufacturing Co.), with the second electrode. The layers were held together by rubber bands and placed in a chamber containing 20 mM Tris base, 150 mM glycine, and 20% (vol/vol) methanol. Residual-binding sites on the paper were blocked by incubation for 40 min at 37°C in a solution containing 0.9% NaCl, 10 mM Tris-HCl at pH 7.4 (Tris/saline), 3% gelatin (Sigma Chemical Co.), and 0.05% Tween-20. The sheets were transferred to Tris/saline with gelatin and Tween-20 containing the appropriate antibodies and were incubated overnight on a rocking platform at room temperature. After several washes with Tris/saline and Tris/saline containing 0.05% Nonidet P-40, the nitrocellulose papers were incubated with iodinated protein A (Sigma Chemical Co.), according to the procedure of Burnett (1981). The sheets were again rinsed as described above, briefly blotted with paper towels, wrapped in Saran Wrap (Dow Corning Corp., Midland, MI), and exposed at -70°C to Kodak XAR-5 film.

### HPLC Separation of the Tryptic Phosphopeptides and Identification of Phosphoamino Acids

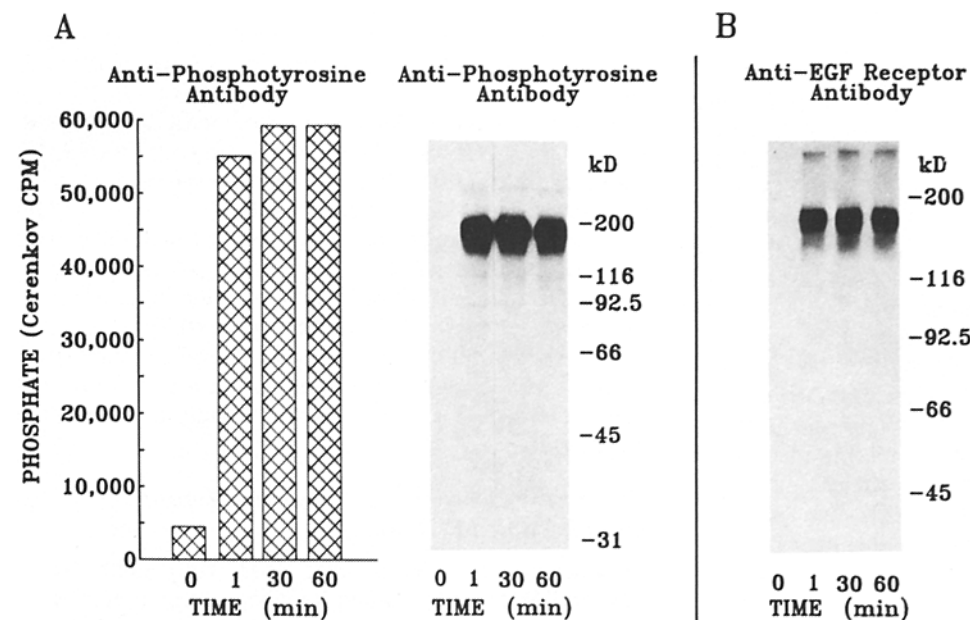
Tryptic phosphopeptides were obtained from the EGF receptor contained in polyacrylamide gel fragments as previously described (Kasuga et al.,

1985; White et al., 1985). The phosphopeptides were separated with HPLC system (Waters Instruments, Inc., Rochester, MN) equipped with a wide-pore RP-318 reverse-phase column (Bio-Rad Laboratories). Phosphopeptides applied to the column were eluted at a flow rate of 1.1 ml/min with a mobile phase composed of water and 0.05% trifluoroacetic, and increasing concentration of acetonitrile between 5 and 25% during 85 min. Fractions (1 ml) were collected in 1.5-ml polypropylene microfuge tubes and the radioactivity in each tube was measured as Cerenkov radiation using a scintillation counter (LKB Instruments, Gaithersburg, MD) with an efficiency of 40%. The percentage of acetonitrile in each sample had no effect on the efficiency of the Cerenkov radiation. Trypsin digestion of the polyacrylamide gel fragments ordinarily released 85% of the radioactivity. About 85% of the radioactivity in the trypsin digest was routinely recovered from the reverse-phase HPLC column. The phosphoamino acids were identified in tryptic peptides by a modification (White et al., 1985) of the method of Hunter and Sefton (1980).

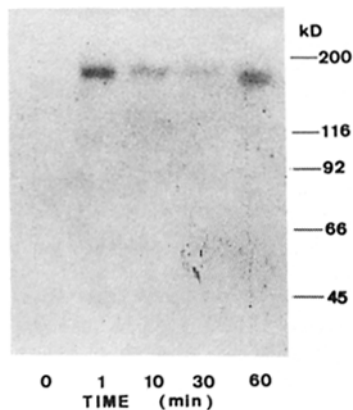
## Results

### Immunoprecipitation of Phosphotyrosine-containing Protein from Crude Extracts of A-431 Cells

To determine the nature of the phosphotyrosine-containing proteins in A-431 cells during EGF stimulation, cells were labeled for 2 h with [ $^{32}$ P]orthophosphate and incubated with 100 nM EGF for 1, 30, or 60 min at 37°C. The labeled proteins were then extracted with SDS, precipitated with  $\alpha$ PTyr, and analyzed by SDS-PAGE. Before the addition of EGF, a small amount of radioactivity was immunoprecipitated from the whole cell extract by the  $\alpha$ PTyr (Fig. 1 A, left), but separation of this material on a 10% resolving gel by SDS-PAGE revealed no discrete phosphoproteins (Fig. 1 A, right). Within 1 min after EGF stimulation, the amount of [ $^{32}$ P]-phosphate immunoprecipitated by the  $\alpha$ PTyr was increased by more than 10-fold, and this remained elevated during the 1-h stimulation. Separation of the proteins immunoprecipitated by the  $\alpha$ PTyr during EGF stimulation indicated that at all times the major phosphoprotein had an  $M_r$  of 170 kD (Fig. 1 A, right) consistent with the known molecular mass of the EGF receptor (Carpenter and Cohen, 1979). Other



**Figure 1.** Immunoprecipitation of phosphotyrosine-containing proteins from EGF-stimulated A-431 cells. Confluent A-431 cells were labeled for 2 h with [ $^{32}$ P]orthophosphate and then stimulated with 100 nM EGF for the indicated time intervals. (A) The phosphotyrosine-containing proteins were immunoprecipitated with 3  $\mu$ g of the  $\alpha$ PTyr and eluted from the precipitate with 50 mM *p*-nitrophenyl phosphate. The total in each eluate is shown on the left. The eluted proteins were reduced with 100 mM DTT and separated by SDS-PAGE and identified by autoradiography (A, right). (B) The proteins were eluted from the  $\alpha$ PTyr with 50 mM *p*-nitrophenylphosphate and reimmunoprecipitated with the  $\alpha$ EGFR, reduced with DTT, and separated by SDS-PAGE. The autoradiogram is shown.



**Figure 2.** Immunoblot analysis of extracts of A-431 cells with  $\alpha$ PTyr. A-431 cells incubated in the absence or presence of EGF (100 nM) for 1, 10, 30, and 60 min at 37°C, were lysed, loaded on an SDS-7.5% polyacrylamide, transferred to nitrocellulose paper, and allowed to react with  $\alpha$ PTyr. Markers are indicated on the right.

phosphoproteins were observed during the EGF stimulation with relative molecular masses of 120, 90, and 70 kD, respectively. In some experiments, a minor band at 36 kD was also observed. These minor components constituted less than 5% of the total. Similar results were obtained by immunoblot analysis with  $\alpha$ PTyr (Fig. 2) providing further evidence that the antibody mostly reacted with the EGF receptor.

To verify that the 170-kD protein was the EGF receptor, the labeled proteins were eluted from the  $\alpha$ PTyr using 50 mM *p*-nitrophenyl-phosphate, then reprecipitated with an  $\alpha$ EGFR. All of the 170-kD protein which had been eluted from the  $\alpha$ PTyr immunoadsorbant was reprecipitated with  $\alpha$ EGFR and migrated with an  $M_r$  of 170 kD during SDS-PAGE (Fig. 1 *B*). The other minor proteins were not precipitated by this antibody. Thus the major phosphoprotein immunoprecipitated by  $\alpha$ PTyr during EGF stimulation of the A-431 cells was the EGF receptor.

#### **Localization of the Tyrosine-phosphorylated EGF Receptor in A-431 Cells by Immunofluorescence with $\alpha$ PTyr**

Since the major protein that reacted with the  $\alpha$ PTyr during EGF stimulation of A431 cells was the EGF receptor, this antibody could be used to follow the movement of the phosphorylated receptor during EGF stimulation. A-431 cells cultured for 12 h in the absence of serum and EGF were fixed, permeabilized by a cycle of dehydration and rehydration, incubated with  $\alpha$ PTyr, and stained with FITC-conjugated goat anti-rabbit IgG. No immunofluorescence was observed in accord with our finding that no phosphotyrosine-containing proteins were immunoprecipitated from unstimulated cells (Fig. 3 *a*). By contrast if the fixation step was preceded by a 2-h incubation at 4°C with 100 nM EGF, an immunofluorescent reaction was evident in most cells of the culture consistent with labeling of the plasma membrane (Fig. 3 *b*). A comparable immunofluorescent action was observed when EGF was added to A-431 cells maintained at 37°C for only 1 min (Fig. 3 *c*). Changing the plane of focus indicated that microvilli and surface projections were preferentially labeled (Fig. 3 *d*).

The specificity of the  $\alpha$ PTyr reaction was determined by measuring the ability of phosphoaminoacids and phosphotyramine to inhibit the immunofluorescent reaction. EGF-stimulated immunofluorescence was completely inhibited when A-431 cells preincubated with  $\alpha$ PTyr were subsequently incubated for 45 min with phosphotyrosine (2 mM) or its analogue phosphotyramine (2 mM). As expected, no inhibition was observed when 2 mM phosphoserine or phosphothreonine were present indicating that the  $\alpha$ -PTyr reacted specifically with phenylphosphate moieties (data not shown). Likewise, immunofluorescence was absent in unpermeabilized EGF-stimulated cells indicating that the phosphotyrosine residues occurring during EGF stimulation were located inside of the cells. These results are consistent with the orientation of the EGF receptor deduced from its primary amino acid sequence (Ullrich et al., 1984).

Thus, during the initial response of A-431 cells to EGF binding, the EGF receptor at the plasma membrane was the predominant protein to undergo tyrosine phosphorylation. Parallel incubations in the presence of insulin (100 nM) or PMA (50 nM) did not induce an immunofluorescent reaction with  $\alpha$ PTyr. This is not surprising since, when compared with the EGF receptor, A-431 cells possess only a small number of insulin receptors, almost certainly below the level of sensitivity of the immunofluorescent technique, and PMA has been shown to stimulate threonine, rather than tyrosine, phosphorylation of the EGF receptor (Cochet et al., 1984; Downward et al., 1985; King and Cooper, 1986). Moreover, as previously demonstrated by biochemical techniques, a 40-min preincubation at 37°C with PMA (50 nM) significantly reduced EGF-induced tyrosine phosphorylation (data not shown).

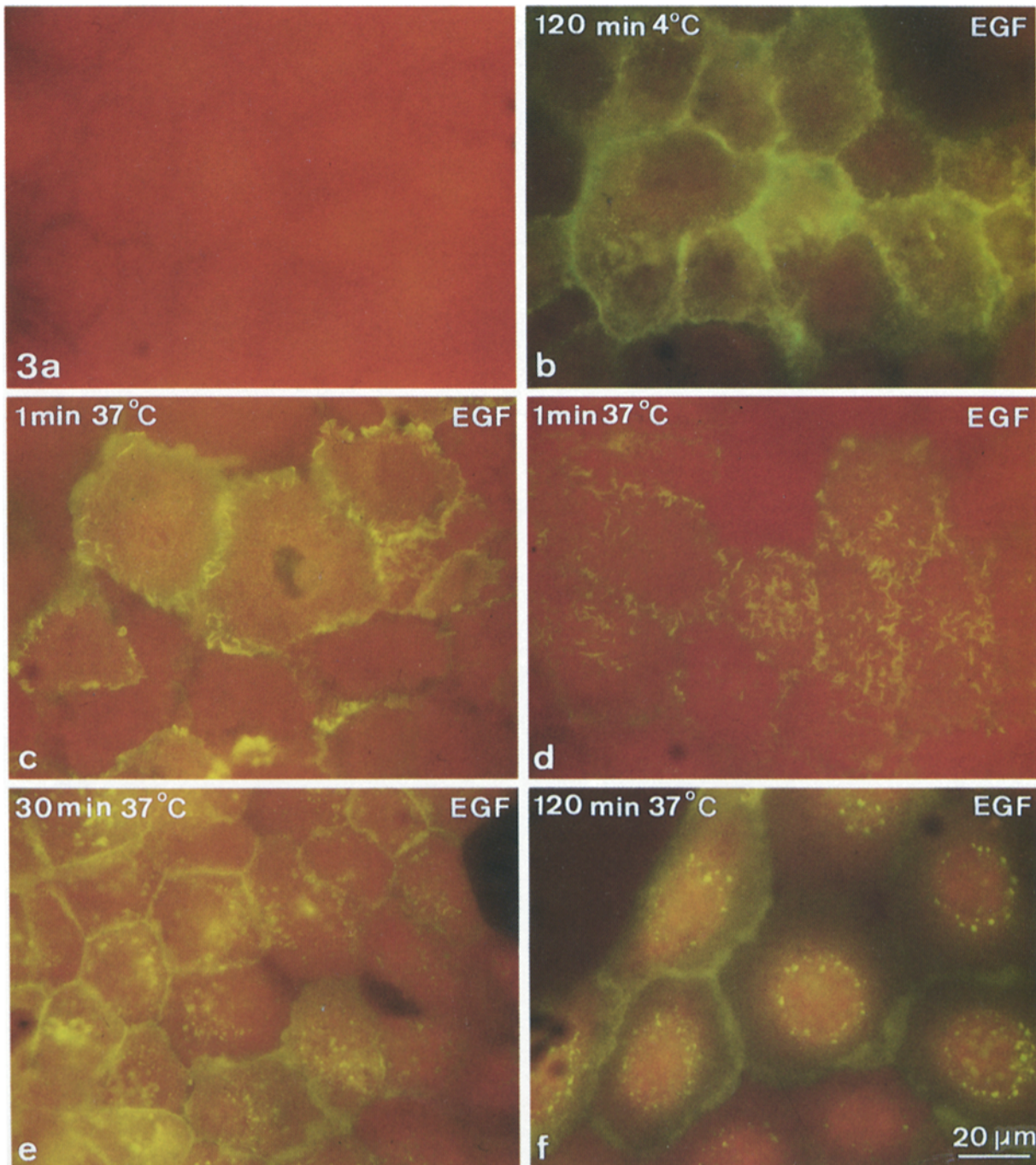
#### **The Fate of the Phosphorylated EGF Receptor**

To determine the fate of the phosphorylated EGF receptor, A-431 cells were incubated for various periods of time at 37°C in the presence of EGF. By 30 min the fluorescent reaction, which was of approximately the same intensity as after 1 min, appeared as intracytoplasmic dots in addition to the original plasma membrane delineation (Fig. 3 *e*). This pattern persisted after 120 min at 37°C (Fig. 3 *f*). In regions of the culture where A-431 cells were flattened, these positive dots were grouped in the perinuclear regions of the cells (Fig. 3 *f*).

To verify the intracellular location of the EGF receptor after 60 min of EGF stimulation, semi-thin sections of A-431 cells were incubated with the  $\alpha$ PTyr followed by fluorescent goat anti-rabbit IgG. This confirmed the presence of EGF-induced PTyr residues not only associated with the plasma membrane as by 1 min of incubation (Fig. 4 *a*) but also intracellularly located, as positive dots in the perinuclear region of the cytoplasm (Fig. 4 *b*). Since the  $\alpha$ PTyr recognized almost exclusively the EGF receptor after EGF stimulation (Figs. 1 and 2), the intracellular fluorescence almost certainly represents internalized tyrosine-phosphorylated EGF receptors.

#### **Electron Microscopic Localization of Tyrosine-phosphorylated EGF Receptors**

Thin sections of aldehyde-fixed A-431 cells embedded in Lowicryl K4M were studied by electron microscopy after

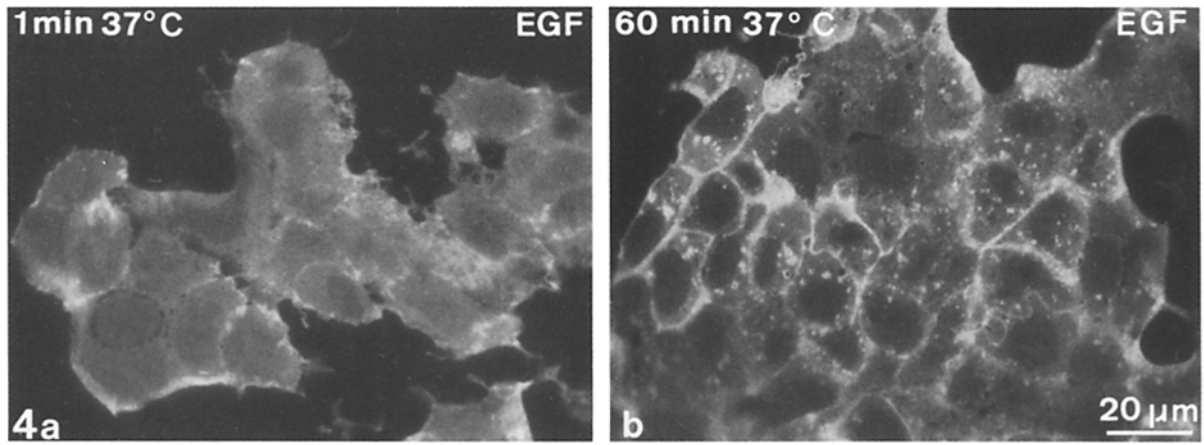


**Figure 3.** Immunofluorescence staining with the  $\alpha$ PTyr of A-431 cells fixed with 4% paraformaldehyde/0.1% glutaraldehyde and permeabilized by dehydration rehydration. Cells were cultured in the absence of FCS for 12 h and fixed (*a*) or further incubated for various periods of time at 4°C or 37°C in the presence of 100 nM EGF. After 2 h of incubation at 4°C or 1 min of incubation at 37°C in the presence of EGF, the fluorescent reaction delineate the plasma membrane of most cells (*b* and *c*). Changing the plane of focus indicates that surface projections are preferentially labeled (*d*). At prolonged incubation times at 37°C, the fluorescent reaction appears largely as intracytoplasmic dots in addition to the original plasma membrane delineation (*e* and *f*).

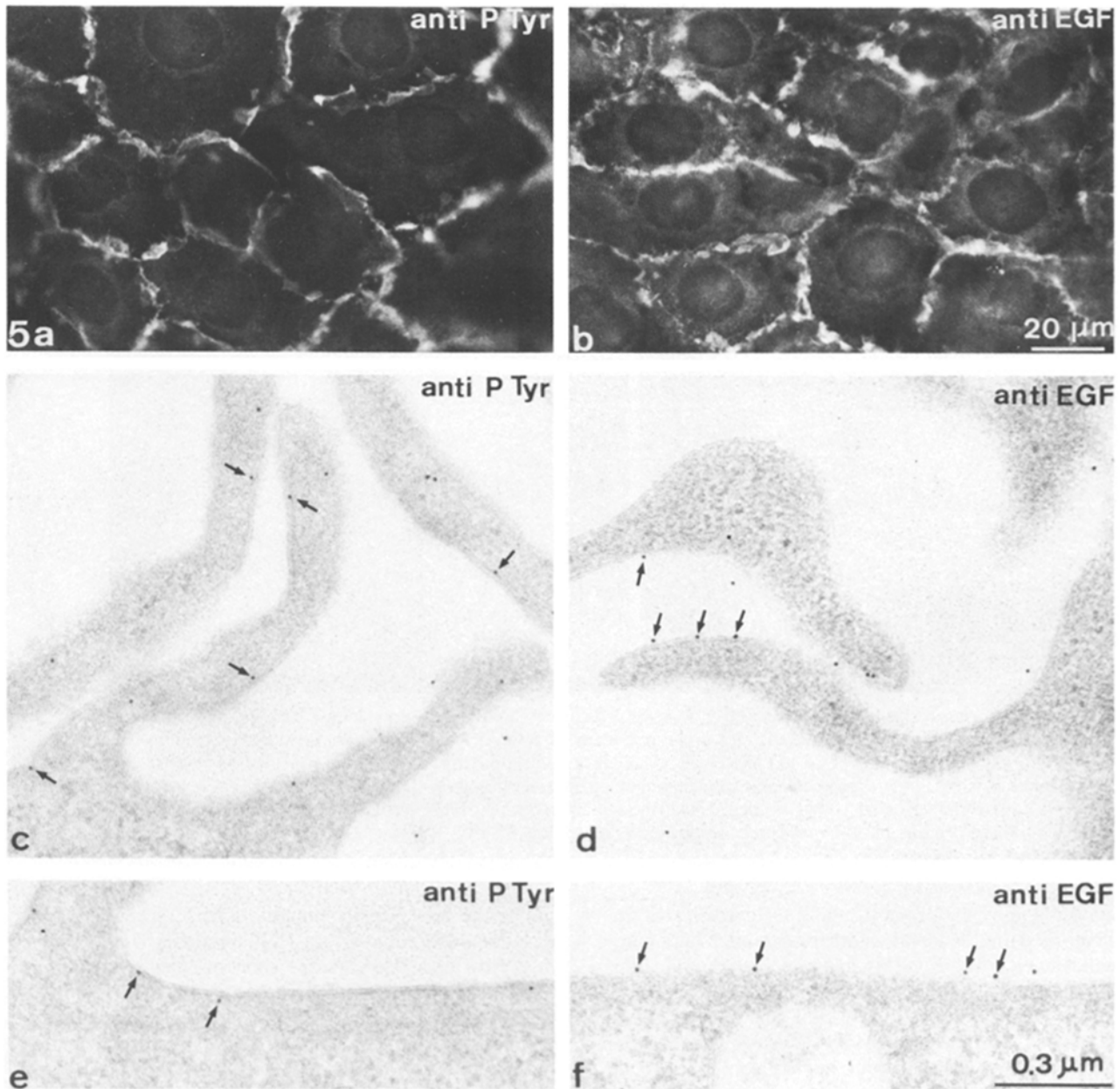
immunostaining by the protein A-gold technique. This approach allowed precise identification of the subcellular compartments labeled by the  $\alpha$ PTyr and quantitative evaluation of the intensity of immunolabeling in these compartments.

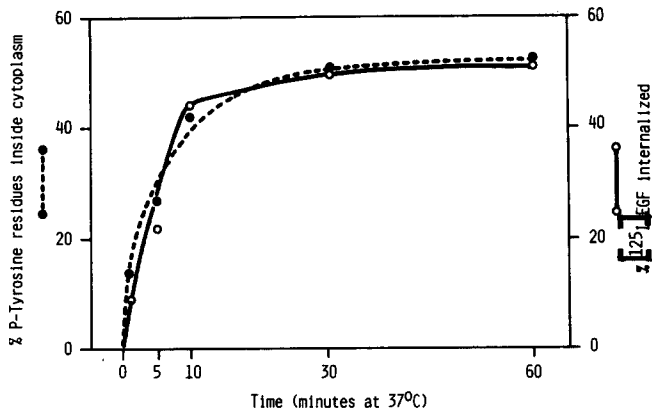
In A-431 cells exposed to EGF for 1 min at 37°C, more than 85% of EGF-induced phosphotyrosine residues were associated with the plasma membrane, more precisely on the

cytoplasmic side of that membrane (Fig. 5). These antigenic sites were preferentially located in microvilli (1.32 sites per  $\mu\text{m}$  microvilli vs. 0.79 sites per  $\mu\text{m}$  nonvillous segments). At no time was there a significant association of the gold particles with the coated pits. However, the Lowicryl K4M embedding technique is not the most appropriate technique for a quantitative analysis of these structures. During pro-



**Figure 4.** Comparison of the immunofluorescent staining obtained with  $\alpha$ PTyr on semi-thin sections of A-431 cells incubated for 1 min (a) or 60 min (b) at 37°C in the presence of EGF (100 nM).





**Figure 6.** Comparison of the internalization of EGF-induced phosphotyrosine residues and of [ $^{125}$ I]EGF in A-431 cells. The internalization of EGF-induced phosphotyrosine residues was estimated by quantifying the proportion of the total number of A-431 cells associated gold particles that were found inside the cell at each time point studied. The internalization of [ $^{125}$ I]EGF was determined by quantitative EM autoradiography. Autoradiographic grains  $>250$  nm beyond the plasma membrane were considered inside the cells.

longed exposure to EGF at 37°C, the density of gold particles associated with the microvilli of the plasma membrane decreased by 40% to a mean of 0.80 sites per  $\mu\text{m}$ .

Parallel to this redistribution of EGF-induced phosphotyrosine residues on the surface of A-431 cells, the EGF receptor was progressively internalized (Figs. 6 and 7). Internalization at 37°C was rapid and almost maximal by 10 min, with 50% of the phosphorylated EGF receptor being internalized (Fig. 6). Inside the cytoplasm, two compartments were labeled: a tubulo-vesicular system and lysosome-like structures mostly composed of multivesicular bodies (Fig. 7, *c* and *e*). As previously described (Tran et al., 1986), these two compartments are closely related. In both cases gold labeling occurred on the cytoplasmic side of the limiting membrane (Fig. 7, *c* and *e*). A quantitative analysis of the labeling of these two intracellular compartments as a function of incubation time at 37°C showed that labeling of the tubulo-vesicular system reached a maximum after 1 min of incubation whereas labeling of multivesicular bodies required nearly 30 min to reach steady-state (Fig. 8, *A* and *B*). At all time intervals, the labeling of the mitochondria and nucleus remained at minimal values similar to those observed in the absence of EGF (Fig. 8, *A* and *B*).

#### **Do EGF-induced Phosphotyrosine Residues Follow the Same Intracellular Pathway in A-431 Cells As EGF Itself?**

To compare the fate of the EGF-induced phosphotyrosine residues with the pathway followed by the ligand itself, we

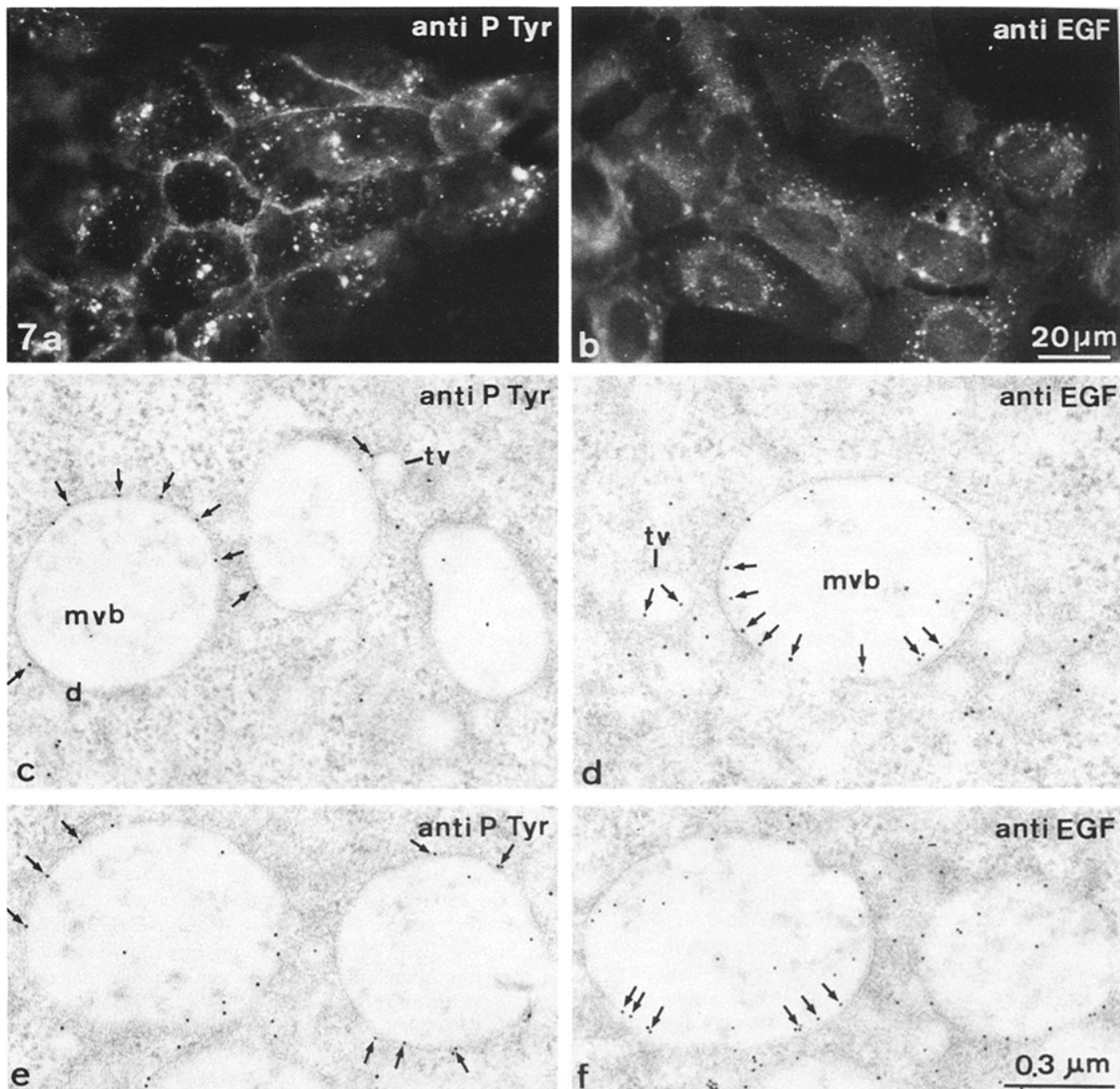
have analyzed by quantitative EM autoradiography the distribution of [ $^{125}$ I]EGF bound to A-431 cells under the same experimental conditions as described above. During incubation at 37°C, [ $^{125}$ I]EGF was internalized in A-431 cells at the same rate as EGF-induced phosphotyrosine residues (Fig. 6). Once internalized the labeled ligand was associated with the same intracellular compartments as phosphotyrosine residues; i.e., the tubulo-vesicular structures and lysosomal structures (Fig. 9). Furthermore, like the phosphorylated EGF receptor, [ $^{125}$ I]EGF was first associated with the tubulo-vesicular compartment and later this radioactivity was transferred to lysosomal structures (Fig. 9).

As a second approach for following the fate of the EGF ligand, cell bound EGF was localized with a polyclonal anti-EGF antibody ( $\alpha$ EGF) followed by immunofluorescent labeling with FITC-conjugated goat anti-rabbit (IgG) or immunocytochemical labeling with protein A-gold. As determined by immunofluorescence, EGF bound to A-431 cells initially at the cell surface and then was progressively internalized as a function of incubation time at 37°C. At all time points studied, the immunofluorescent reactions showed a similar distribution pattern for both EGF-induced tyrosine phosphorylation and EGF itself (Figs. 5 and 7). After internalization, the tubulo-vesicular structures and lysosomal structures (mostly multivesicular bodies) were preferentially labeled with the  $\alpha$ EGF. As expected, labeling was observed on the luminal side of the vacuoles in opposition to the external (or cytoplasmic) labeling obtained with  $\alpha$ PTyr (Fig. 7).

#### **Phosphorylation Sites on the EGF Receptor during Prolonged Incubation of A-431 Cells with EGF**

To determine whether structural changes occur in the phosphotyrosine-containing EGF receptors during their internalization and association with the intracellular compartments, we carried out tryptic peptide mapping of the phosphorylated receptor.  $^{32}\text{P}$ -labeled A-431 cells were stimulated with 100 nM EGF for 1, 10, 30, or 60 min and the phosphorylated receptor was purified by sequential immunoprecipitation with  $\alpha$ PTyr and  $\alpha$ EGFR. After identification of the receptor by SDS-PAGE and autoradiography, the protein was completely digested with excess trypsin and the phosphopeptides were separated by reverse-phase HPLC. The chromatogram shown in Fig. 10 revealed three distinct peaks of radioactivity labeled P1, P2, and P3. Phosphoamino acid analysis of these fractions showed that they contained only phosphotyrosine residues. All of the minor peaks also contained only phosphotyrosine except for those eluting at 27 and 53 min which contained both phosphoserine and phosphotyrosine; however, no phosphothreonine was detected in any of the phosphopeptides. Thus, there appear to be three major sites of phosphorylation of the EGF receptor, all are almost entirely phosphotyrosine, and phosphorylation is stable during 1 h of EGF stimulation.

**Figure 5.** Immunofluorescent and immunocytochemical localization of EGF-induced phosphotyrosine residues (*left*) and EGF (*right*) at the plasma membrane of A-431 cells incubated for 1 min (*a-d*) or 30 min (*e* and *f*) at 37°C in the presence of EGF. (*a* and *b*) The immunofluorescent reaction localizes phosphotyrosine residues and EGF to the plasma membrane of the A-431 cells. (*c* and *d*) The protein A-gold labeling (*arrows*) of microvilli and surface projections of A-431 cells is located on the cytoplasmic side of the membrane when  $\alpha$ PTyr is used (*c*) and on the exoplasmic side of the membrane when the  $\alpha$ EGF is applied (*d*). (*e* and *f*) After prolonged incubation periods (30 min) at 37°C in the presence of EGF the remaining plasma membrane labeling is prominent in nonvillous segments. The  $\alpha$ PTyr (*e*) and the  $\alpha$ EGF (*f*) show a reverse distribution pattern around the plasma membrane ( $\alpha$ PTyr inside;  $\alpha$ EGF outside) (*arrows*).

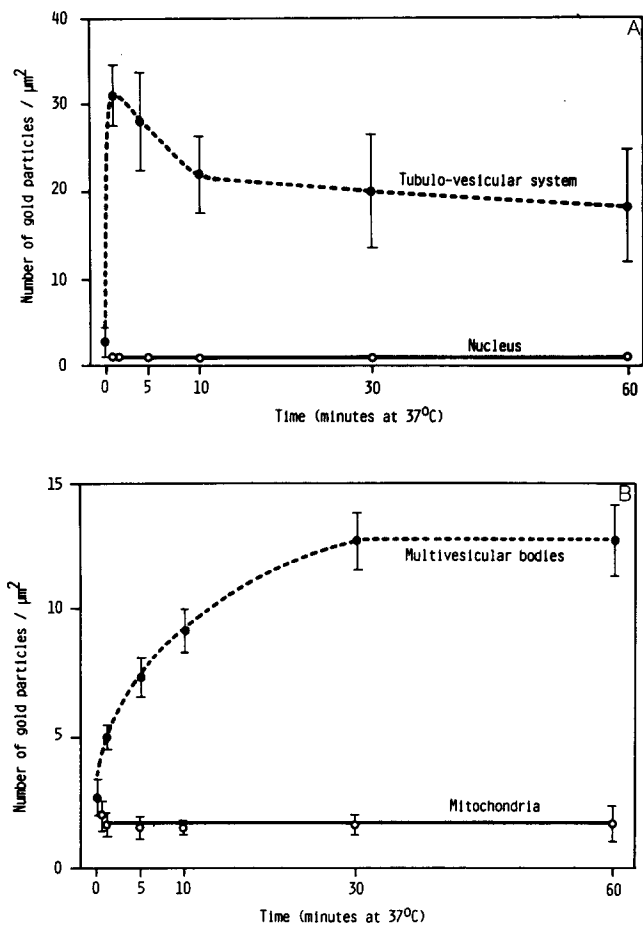


**Figure 7.** Immunofluorescent and immunocytochemical localization of EGF-induced phosphotyrosine residues and EGF inside A-431 cells incubated for 30–60 min at 37°C in the presence of EGF. (*a* and *b*) The immunofluorescent reaction localizes phosphotyrosine and EGF as small dots inside A-431 cells. (*c* and *d*) Representative examples of lysome-like structures (multivesicular bodies [*mvb*] and tubulo-vesicular (*tv*) system labeled with  $\alpha$ P-Tyr (*c*) or with  $\alpha$ EGF (*d*). In *c*, the section of A-431 cells incubated with  $\alpha$ P-Tyr shows gold particles associated with the cytoplasmic leaflet of the limiting membrane of both multivesicular bodies and tubulo-vesicular structures (*arrows*). In *d*, a section incubated with  $\alpha$ EGF shows gold particles associated with the luminal side of the limiting membrane. (*e* and *f*) Consecutive serial sections of two adjacent multivesicular bodies incubated either with  $\alpha$ P-Tyr (*e*) or  $\alpha$ EGF (*f*). Arrows point at gold particles associated with the cytoplasmic side of the limiting membrane when  $\alpha$ P-Tyr is used. Note that numerous gold particles associated with the luminal side of the same limiting membrane when  $\alpha$ EGF is used. Numerous gold particles associate also with small vesicles inside mvbs.

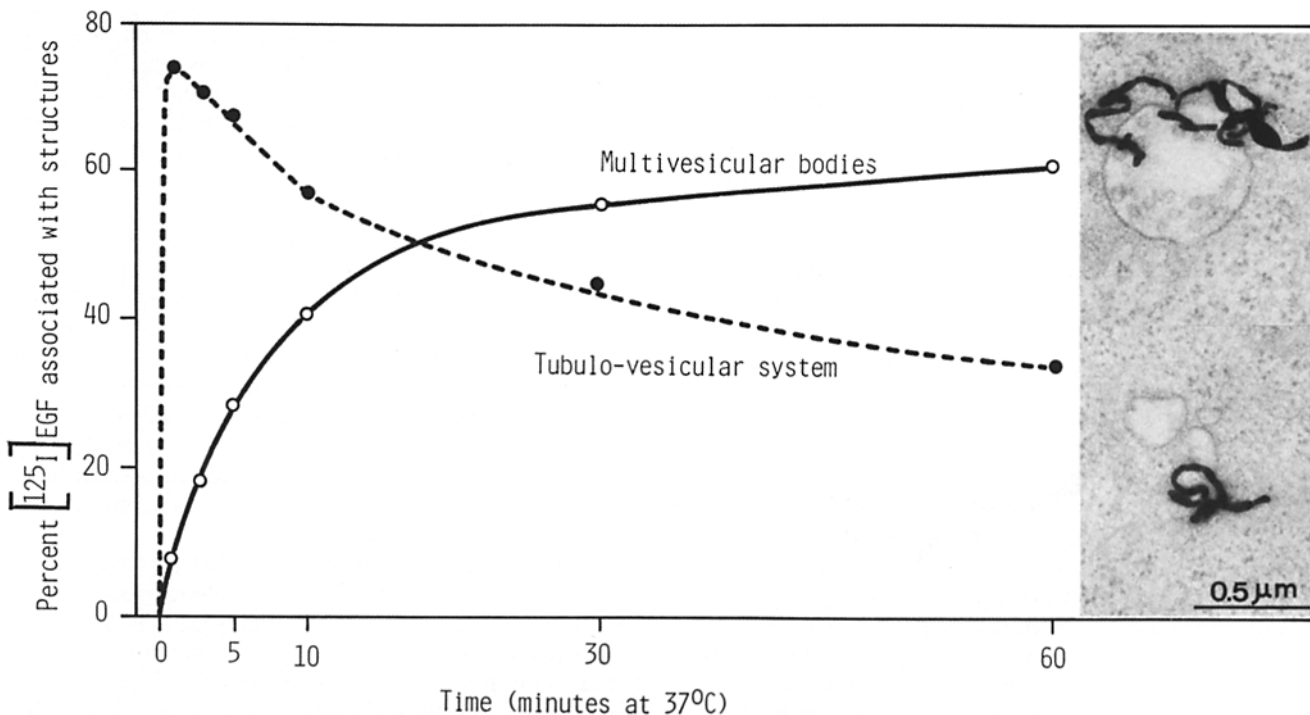
## Discussion

In this report, we describe the movement of the EGF receptor into the intracellular compartments of A-431 cells after EGF-stimulated tyrosine autophosphorylation. On the basis of the data presented, one can draw three major conclusions. (*a*) After EGF-induced tyrosine autophosphorylation at the cell surface, the occupied EGF receptors remain phosphorylated (activated) for at least 60 min; they are progressively internalized and associate with various intracellular

structures; i.e., tubulo-vesicular system and multivesicular bodies. (*b*) During this internalization process the EGF receptor remains intact and neither the EGF receptor nor any of its phosphorylated fragments associate with the nucleus. (*c*) On average,  $\sim 50\%$  of the phosphorylated EGF receptors are present at the plasma membrane level and remain on the cell surface throughout a 1-h incubation suggesting that tyrosine phosphorylation alone is not sufficient as a signal for internalization.



**Figure 8.** Concentration of phosphotyrosine residues in the tubulo-vesicular system and the nucleus (A) and the multivesicular bodies and mitochondria (B) as a function of incubation time at 37°C in the presence of EGF.



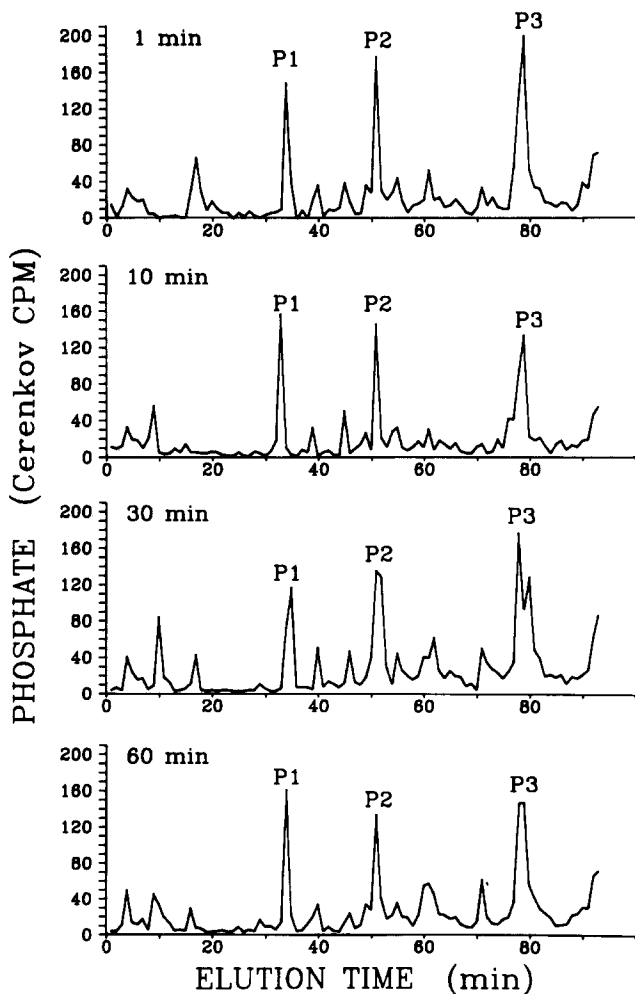
**Figure 9.** Percentage of the total number of autoradiographic grains found inside A-431 cells that are associated with the tubulo-vesicular system and multivesicular bodies in the course of [<sup>125</sup>I]EGF-binding at 37°C. (Inset) Representative examples of a multivesicular body (upper right) and a tubulo-vesicular system (lower right).

Although the initial interaction of EGF with its receptor occurs at the cell surface, the finding that the phosphorylated EGF receptor is internalized suggests that endocytosis of the EGF receptor provides a mechanism to translocate activated receptor kinase from the plasma membrane to the cell interior where it might be the starting point of a cascade of phosphorylation of plasma membrane-inaccessible substrates as suggested at the biochemical level by Cohen and Fava (1985). In this manner, EGF-induced internalization of the EGF receptor could be of physiological significance in controlling some of the cellular responses to EGF.

Inside the cells, the intact tyrosine-phosphorylated EGF receptor was found associated with tubulo-vesicular structures as well as with multivesicular bodies, but not with the nucleus or associated structures. These are also the major sites of localization of the EGF receptors detected with a battery of αEGFRs (Carpentier et al., 1986). A similar exclusion from the nucleus was also noted for the kinase domain and the external segment of the EGF receptor in other cell types (Beguinet et al., 1986; Miller et al., 1986). Within the resolution of our current study, no specific association of phosphorylated EGF receptor with the Golgi region was observed, as has been reported for the EGF ligand by Willingham and Pastan (1982).

Our observations clearly show that in A-431 cells the tyrosine-phosphorylated EGF receptor does not dissociate from the ligand in the tubulo-vesicular compartment (endosome) and that the phosphorylated receptors have no detectable degradation over a 1-h time course at 37°C. This is in agreement with the data of Stoscheck and Carpenter (1984a, b) who showed that the half life of the occupied EGF receptor is 7.4-fold longer in A-431 cells than in fibroblasts.

The exact nature of the protein substrates of the EGF receptor kinase remains uncertain. Although our αTyr



**Figure 10.** Separation of tryptic phosphopeptides from the EGF receptor by HPLC. Confluent A-431 cells were labeled for 2 h with [ $^{32}$ P]orthophosphate and then stimulated with 100 nM EGF for 1, 10, 30, and 60 min at 37°C. The EGF receptor was purified by sequential immunoprecipitation with the  $\alpha$ PTyr and  $\alpha$ EGFR, and analyzed by SDS-PAGE. The gel fragment containing the phosphorylated receptor was then digested with trypsin and the tryptic phosphopeptides were separated on an RP-318 reverse-phase column.

recognizes some proteins of lower molecular mass than the EGF receptor which are phosphorylated on tyrosine residues in EGF-treated A-431 cells, including lipocortin ( $M_r = 36$  kD) (Fava and Cohen, 1984) as well as a 70- and a 90-kD band (Frackelton et al., 1983), these phosphoproteins are very minor when compared to the receptor itself. These data are consistent with previous studies which have shown that lipocortin phosphorylation occurs relatively slowly after EGF binding and is not maximal for at least 4 h (Pepinsky and Sinclair, 1986); thus lipocortin is not a likely candidate to be involved in the early part of the phosphorylation cascade after internalization. It is possible however that some of these other minor early substrates of the EGF receptor kinase are important in such a cascade. It is also possible that some phosphotyrosine-containing proteins do not react well with the  $\alpha$ PTyr due to folding of the protein or inaccessibility of the phosphotyrosine residues under non-denaturing conditions.

Analysis of the phosphorylation sites in the EGF receptor by tryptic digestion and reverse-phase HPLC revealed three major sites of phosphorylation at all time points. Phosphopeptides corresponding to each site were detected within 1 min after EGF stimulation and were present even after 1 h of incubation at 37°C. Three tyrosine phosphorylation sites in the solubilized EGF receptor of the A-431 cell have been observed previously and identified by amino acid sequence analysis: tyrosines 1,173, 1,148, and 1,068 (Downward et al., 1984). By their relative mobility on HPLC, we have labeled the peptides observed in our system as P1, P2, and P3. Although previous studies have suggested that only P1 and P3 (Cohen et al., 1982; Downward et al., 1984) are increased after EGF stimulation of intact cells, in the current study all three tryptic peptides were stimulated with equal intensity. Since EGF-stimulated phosphorylation occurs in the cytoplasmic domain of the EGF receptor, close to the carboxy terminus (Downward et al., 1984), our results do not support the hypothesis that a cleavage of the receptor occurs to release a phosphorylated fragment that acts as a second messenger (Das and Fox, 1978; Basu et al., 1984).

Phosphorylation of threonine residue 654 of the EGF receptor has been observed in the basal state, in response to phorbol ester treatment (Hunter and Cooper, 1984; Iwashita and Fox, 1984; Davis and Czech, 1985; Downward et al., 1985), and to a lesser and variable extent in response to EGF itself (King and Cooper, 1986). Threonine phosphorylation appears to accelerate the dissociation of EGF (Friedman et al., 1984) thus decreasing the receptor affinity and the ability of EGF to activate the receptor kinase. We find that the subset of receptors reacting with the  $\alpha$ PTyr in our A-431 cells contained less than 5% phosphoserine and no detectable phosphothreonine. Thus, it is possible that the EGF receptor, like the insulin receptor (Pang et al., 1985), exists in discrete pools which differ in phosphorylation state and that the dephospho-forms of the receptor are the primary targets for EGF-stimulated tyrosine phosphorylation, while the threonine/serine forms are inactive or less active.

With  $\alpha$ PTyr we can follow the movement of the subset of EGF receptors that were initially located at the plasma membrane and which respond to EGF with tyrosine phosphorylation. Using various anti-EGF receptor antibodies, we have previously shown by electron microscopy that only 15% of the total EGF receptors in A-431 cells are at the plasma membrane (Carpentier et al., 1986) consistent with the data of King and Cooper (1986). Immediately after EGF stimulation, however, the  $\alpha$ PTyr recognized only the plasma membrane EGF receptors. The intracellular receptors were not recognized by  $\alpha$ PTyr because they are not accessible to EGF and hence did not undergo tyrosine autophosphorylation. Furthermore, since receptor phosphorylation reached steady state within 1 min after EGF binding, it appears that the large fraction of intracellular receptors did not undergo de novo tyrosine phosphorylation, even at later times.

Although the EGF receptors were rapidly tyrosine phosphorylated ( $t_{1/2} < 1$  min) and rapidly internalized ( $t_{1/2} = 5$  min) after EGF binding, after 10 min  $\sim 50\%$  of the phosphorylated receptors were still plasma membrane associated and this level remained constant for 60 min. Thus, tyrosine phosphorylation alone does not appear to be a sufficient signal to induce internalization. However, our data do not exclude the possibility that all tyrosine-phosphorylated recep-

tors at some time become internalized and that they either recycle to the membrane with their tyrosine phosphate residues intact or are replaced by nonphosphorylated EGF receptors which are rapidly stimulated by EGF and undergo de novo tyrosine phosphorylation. Studies using in vitro mutagenesis have suggested that the intracellular portion of the EGF receptor is required for normal internalization, but that internalization may occur after deletion of a COOH-terminal domain containing two tyrosine-phosphorylation sites (Livneh et al., 1986).

The understanding of EGF effects on cell growth requires further elucidation of the steps between EGF activation of the receptor kinase and nuclear signalling. Although activation of transcription occurs shortly after exposure of cells to EGF (Murdoch et al., 1982; Muller et al., 1984; Elder et al., 1984), no information is available to indicate how this response might be triggered. In A-431 cells which provide the most extensively studied model of EGF action (Cohen and Fava, 1985; Stoscheck and Carpenter, 1983, 1984; Downward et al., 1984; Cohen et al., 1980; Hunter and Cooper, 1981; Fava and Cohen, 1984; Weber et al., 1984; Ullrich et al., 1984; Bertics and Gill, 1985), EGF stimulates cell growth at low concentration, but inhibits cell growth at high concentration (Gill and Lazar, 1981; Kawamoto et al., 1983). Within the limits of detection of the morphological method used the tyrosine-phosphorylated EGF receptor or a fragment of the receptor does not appear to enter the nucleus. The possibility remains, however, that through internalization of the tyrosine-phosphorylated, activated, occupied EGF receptor, a cascade of phosphorylation reactions involving other kinases and/or phosphatases is initiated, ultimately resulting in activation of the cellular substrates responsible for modifying cell growth at the level of the nucleus.

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