

# Perinuclear Location and Recycling of Epidermal Growth Factor Receptor Kinase: Immunofluorescent Visualization Using Antibodies Directed to Kinase and Extracellular Domains

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**Abstract.** This paper describes studies on the migratory behavior of epidermal growth factor (EGF) receptor kinase using antibodies that are specific for either the kinase domain or the extracellular domain of the receptor. Antiserum was raised to a 42,000-D subfragment of EGF receptor, which was shown earlier to carry the kinase catalytic site but not the EGF-binding site. Another antiserum was raised to the pure intact 170,000-D EGF receptor. The specificities of these antibodies were established by immunoprecipitation and immunoblotting experiments. The domain specificity was examined by indirect immunofluorescent staining of fixed cells. The anti-42-kD peptide antibody could bind specifically to EGF receptors of both human and murine origin and was found to be directed to the cytoplasmic part of the molecule. It did not bind to EGF receptor-negative cells, which contained other types of tyrosine kinases. The antibodies raised against the intact receptor recognized only EGF receptor-

specific epitopes and were directed to the extracellular part of the molecule.

The anti-receptor antibodies described above were used to visualize the cyclic locomotory behavior of EGF receptor kinase under various conditions of EGF stimulation and withdrawal. The receptor was examined in fixed and permeabilized cells by indirect immunofluorescent staining. The results demonstrate the following: (a) the receptor kinase domain migrates to the perinuclear region upon challenge with EGF; (b) both extracellular and cytoplasmic domains of the receptor are involved in migration as a unit; (c) withdrawal of EGF results in rapid recycling of the perinuclear receptors to the plasma membrane; (d) this return to the cell surface is inhibited by methylamine, chloroquine, and monensin; and (e) neither the internal migration nor the recycling process is blocked by inhibitors of protein biosynthesis.

**T**HE epidermal growth factor (EGF)<sup>1</sup> receptor is a plasma membrane protein that has been implicated in the regulation of cell proliferation and cancer causation (17). It is a single chain polypeptide of 170,000–180,000 D that has an externally facing EGF-binding domain and a cytoplasmically facing tyrosine-specific protein kinase site (10, 13, 41). Although no significant biological function has yet been assigned to the tyrosine kinase site, the importance of tyrosine kinases in biology is apparent in the stringent conservation of these molecules through prolonged evolutionary times, and in the remarkable homologies seen between different tyrosine kinases in their catalytic domains (25).

Some of the questions that can be asked regarding receptor tyrosine kinases are as follows: (a) What is the molecular basis for growth factor-induced activation of the receptor kinase site? (b) What is the subcellular site of action of the receptor kinase? Is it at the plasma membrane or elsewhere? If the site of action is other than plasma membrane, what

structural features control the migration to the inside of the cell?

We have answered the first question to some extent. Our *in vitro* studies provide experimental evidence for the supposition that the EGF receptor is an allosteric enzyme that can interconvert between active (monomeric) and inactive (dimeric) forms, and that EGF, the ligand, activates the kinase by shifting the equilibrium towards the catalytically active monomeric form (6). These observations on the activation/inactivation cycle of the kinase site led us to the studies described here.

Receptors for many ligands, such as asialoglycoproteins, transferrin, and low density lipoprotein, are known to display a characteristic migratory behavior (3, 9, 22). After binding to their ligands, these receptors cluster or segregate in coated pits and then internalize. Within internal organelles (endosomes), the receptor dissociates from its ligand, and the free receptor recycles back to the cell surface. This round-trip itinerary serves to accumulate the ligand (often a nutrient) inside the cell.

EGF bound to cell-surface receptor is also known to un-

1. *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EBSS, Earle's balanced salt solution.

dergo a rapid endocytic fate (14, 23, 29, 30). The receptor is co-internalized and is subjected to slow proteolysis ( $t_{1/2} > 1$  h) (4, 14, 16, 19, 38). Since EGF is not a simple nutrient, what could be the biological significance of this internal migration of the receptor? It is thought that this process serves a regulatory function by mediating rapid lysosomal destruction of excess EGF and receptor. However it is likely that this migratory process may have yet another biological role that is more closely related to the mitogenic function of EGF receptor. This is suggested by the fact that the EGF receptor is an enzyme. If the target substrate for the receptor kinase is located within the interior of the cell, it will be necessary for the receptor and its substrate to come into close contact; one way to achieve this contact would be through receptor internalization.

To visualize the movement of receptor kinase domain during EGF stimulation and withdrawal, we needed to develop an antibody directed to that domain. Recently we isolated, by proteolysis, a 42,000-D receptor domain that retains the kinase catalytic activity of the intact 170-kD EGF receptor but loses EGF-binding and kinase-regulatory functions (2). Here we describe the development of an immunological probe for this human EGF receptor-derived 42-kD tyrosine kinase. The antibody was found to recognize only the cytoplasmic part of EGF receptor and showed no reaction with other receptor kinases. The specificity of this antibody and other anti-EGF-receptor antibodies used here was established by immunoprecipitation and immunoblot analyses. We used these specific antibodies in indirect immunofluorescent staining studies to visualize the subcellular location of EGF receptor kinase under various conditions of stimulation and withdrawal. The results demonstrate that the EGF receptor kinase displays a cyclic locomotion that is controlled by its state of activation. The cycle of locomotion can be disturbed by certain drugs that inhibit receptor mitogenicity but not by inhibitors of protein biosynthesis. Some implications of these findings are discussed.

## Materials and Methods

### Materials

EGF was purified from mouse submaxillary glands (34) and radiolabeled by the chloramine-T procedure (13).  $\gamma$ - $^{32}$ P-ATP was prepared with inorganic  $^{32}$ P (ICN K & K Laboratories Inc., Plainview, NY) and Gamma-Prep A kit (Promega Biotech, Madison, WI) following the manufacturer's instruction. The following reagents were from Sigma Chemical Co. (St. Louis, MO): rhodamine- and fluorescein-conjugated second antibodies, methylamine, monensin, and chloroquine.

### Cell Culture

Human epidermoid A431 carcinoma cells, human fetal lung WI-38 fibroblasts (nonsenescent, a kind gift from Dr. Paul Phillips of Wistar Institute), murine Swiss 3T3 fibroblasts, and NR-6 fibroblasts (EGF receptorless variants of 3T3) were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum and 10  $\mu$ g/ml gentamycin.

### Immunological Tools

**Anti-42-kD Peptide Antibody.** A rabbit antiserum was generated to the pure 42-kD kinase as follows. EGF receptor was solubilized from human A431 plasma membranes and then purified by EGF affinity chromatography as described (2, 6, 15). The pure EGF receptor (50  $\mu$ g) was incubated at 20°C for 30 min with 2  $\mu$ g/ml trypsin (pure, crystalline) in 0.2 ml of 20 mM Hepes, pH 7.4, 5% glycerol, 0.2% Triton X-100 (2). This resulted in the

generation of the 42-kD kinase. After stopping trypsin action with 0.2  $\mu$ mol of *N*-*p*-tosyl-L-lysinechloromethylketone, the mixture was subjected to non-denaturing electrophoresis on 5–20% gradient polyacrylamide gels at pH 8.8 as described earlier (2, 35). After the run, the gel (11-cm long) was cut into 11 slices. The undegraded 170-kD receptor and other EGF-binding forms were recovered in gel slices 1 and 2 (trypsin was also recovered near the top of the gel). The 42-kD peptide was recovered in gel slices 5 and 6 (2). These slices (5 and 6) were homogenized with phosphate-buffered saline (PBS), emulsified with an equal volume of Freund's complete adjuvant, and then injected intradermally at multiple sites into dorsal surfaces of rabbits (New Zealand, Albino, 3–4-mo-old females). The rabbits were reinjected twice at 3-wk intervals with the antigen emulsified in Freund's incomplete adjuvant. 8 d after the last injection, the rabbits were bled. The IgG fraction of the antiserum was purified by protein A-Sepharose column chromatography using 0.1 M glycine-HCl, pH 3.0 as the eluting buffer. All the antibody activity was bound to the column, and >90% was eluted. The eluted IgG was neutralized immediately with Tris, dialyzed extensively against PBS, and stored at  $-70^{\circ}\text{C}$ . This antibody binds specifically to EGF receptors of both human and murine origin, and binds only to receptor cytoplasmic domain (see Results).

**Anti-170-kD Receptor Antibody.** A rabbit antiserum was generated to the intact and pure 170-kD human EGF receptor as described (37). The solubilized and EGF affinity-purified human EGF receptor (2, 6, 15) was subjected to SDS PAGE. The stained gel band containing the 170-kD receptor ( $\sim 40$   $\mu$ g protein) was used to immunize rabbits as described in the previous section. The IgG fraction of the antiserum was purified on protein A-Sepharose columns as described above. The anti-170-kD receptor IgG binds specifically to EGF receptors of both human and murine origin (see Results).

**Murine Antibody Specific for the Human Receptor.** A murine antiserum was obtained after immunization of C57BL/6 mice with a mouse-human somatic hybrid cell line cl 21, which contains human chromosome 7 as the only human chromosome (7, 15). This antiserum has been extensively characterized to react exclusively with the EGF receptor on the cell surface (7, 15). The IgG fraction of this antiserum specifically immunoprecipitates the human EGF receptor, and it inhibits the binding of EGF to the human receptor (15).

### Western Blotting Method

This was done as described (36). Plasma membrane fractions of human A431 cells and mouse 3T3 cells were isolated (6, 39) and subjected to SDS PAGE under reducing conditions. The electrophoretically separated proteins were electrophoretically transferred to nitrocellulose sheets as described by Towbin et al. (40) using a Bio-Rad apparatus (Bio-Rad Laboratories, Richmond, CA). The electrode buffer contained 25 mM Tris/192 mM glycine/20% methanol (vol/vol) at pH 8.3. The run time was  $\sim 10$  h (at 250 mA, 20 V). This ensured efficient transfer of the 170-kD EGF receptor and other high molecular weight proteins. After transfer, the nitrocellulose blots were soaked in 3% bovine serum albumin (BSA), 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4 for 5 h at 20°C to saturate additional protein binding sites. They were then rinsed in saline and incubated at 20°C for 2 h with anti-EGF-receptor rabbit IgG or nonimmune rabbit IgG (0.1–1 mg IgG/ml) in 3% BSA/0.15 M NaCl/10 mM Tris-HCl, pH 7.4. The antibody-treated sheets were washed extensively in saline, then incubated with goat anti-rabbit IgG antibody (100  $\mu$ g/ml) in saline-BSA at 20°C for 2 h, again washed and finally incubated at 20°C for 6 h with  $^{125}\text{I}$ -labeled protein A (10<sup>8</sup> cpm, 500 ng) in saline-BSA. The papers were then washed extensively in saline, dried with a hair dryer, and then subjected to autoradiography.

### Immunoprecipitation of $^{125}\text{I}$ -EGF-Receptor Covalent Complex from Murine Fibroblasts

3T3 or NR-6 cell monolayers in 10-cm dishes were washed with Earle's balanced salt solution (EBSS) containing 20 mM Hepes, pH 7.4 and 1 mg/ml BSA (EBSS-Hepes-BSA), and then incubated at 20°C for 30 min with 30 nM  $^{125}\text{I}$ -EGF (150,000 cpm/ng). Under these conditions the receptor becomes covalently linked to  $^{125}\text{I}$ -EGF (11). The cells containing radiolabeled receptor were washed with EBSS-Hepes and lysed in 200  $\mu$ l of a buffer consisting of 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, 6 mM phenylmethylsulfonyl fluoride, 3% Triton X-100, 5 mM EDTA, 150 mM benzamidine, and 1 U/ml aprotinin. The lysate was frozen-thawed three times, clarified by centrifugation, and then subjected to immunoprecipitation as follows. The lysate was incubated at 4°C for 1 h with rabbit immune IgG or nonimmune IgG (30  $\mu$ g). The immune complexes were isolated by

incubating the mixture at 20°C for 15 min with formaldehyde-fixed *Staphylococcus aureus* (10 mg), and then washing the bacteria with 20 mM Hepes/0.15 M NaCl containing 1 mg/ml BSA and 0.1% Triton X-100. The bound radioactivity was eluted by boiling for 2 min in 1% SDS containing 0.1 M  $\beta$ -mercaptoethanol in 0.15 M Tris-HCl, pH 6.8, and subjected to SDS PAGE and autoradiography using Kodak X-Omat films and DuPont Cronex Lightning Plus intensifying screens (DuPont Co., Diagnostic and Bio-Research Systems, Wilmington, DE) (2, 6, 15).

### Immunoprecipitation Experiments with Crude Extracts of A431 Cells and Mouse Liver Membranes

Plasma membranes (1 mg) from human A431 cells (6, 39) and mouse liver (5) were extracted at 20°C for 30 min with 1% Triton X-100 in 300  $\mu$ l of 20 mM Hepes, pH 7.4, 10% glycerol, 20  $\mu$ g/ml leupeptin. An aliquot of the clarified extract (200  $\mu$ l) was incubated at 4°C for 1 h with 16 mg of *S. aureus* containing bound 60  $\mu$ g of rabbit immune IgG (anti-42-kD peptide antibody or anti-170-kD receptor antibody) or nonimmune IgG, in 60  $\mu$ l of 20 mM Hepes, pH 7.4/10% glycerol. The bacteria were then washed three times with 20 mM Hepes/0.15 M NaCl containing 1 mg/ml BSA and 0.1% Triton X-100 (Hepes/NaCl/BSA/Triton). The washed bacteria were suspended in 40  $\mu$ l of Hepes/NaCl/BSA/Triton containing 1.2 mM MnCl<sub>2</sub> and either no EGF or 1  $\mu$ M EGF. After 10 min at 4°C, phosphorylation was initiated by the addition of 10  $\mu$ l of 25  $\mu$ M  $\gamma$ -<sup>32</sup>P-ATP (100 cpm/fmol). After incubation at 4°C for 30 min, the bacteria were washed, and the bound radioactivity was analyzed by SDS PAGE and autoradiography (2, 6, 15).

### Indirect Immunofluorescent Staining

For these experiments, cells were grown on glass coverslips (1-cm diam) in 24-well multiwells. These were treated with EGF and/or other reagents (see figure legends), and then subjected to either of the three following methods of fixation: (a) Fixation without permeabilization. Cell monolayers on glass coverslips were fixed with 3% paraformaldehyde-PBS for 10 min at 4°C, washed with PBS, and then rid of excess paraformaldehyde by treatment with 50 mM NH<sub>4</sub>Cl-PBS at 4°C for 40 min (20). (b) Fixation followed by Triton X-100 permeabilization. Cell monolayers fixed with paraformaldehyde as in a were incubated at 20°C for 5 min with 0.5% Triton X-100 in 10 mM Hepes, 0.3 M sucrose, 3 mM MgCl<sub>2</sub>, 50 mM NaCl, pH 7.4, and then washed three times with ice-cold PBS (31). (c) Methanol permeabilization. The cell monolayers on glass slides were incubated with absolute methanol at 20°C for 10 min, then washed three times with ice-cold PBS (21).

Coverslips treated as above were rinsed three times with ice-cold DME and incubated at 4°C for 1 h with 100  $\mu$ l of DME containing 40–200  $\mu$ g of rabbit (or mouse) immune IgG or nonimmune IgG. The treated coverslips were washed with DME and then incubated at 4°C for 1 h with 100  $\mu$ l of DME containing rhodamine-conjugated goat anti-rabbit IgG antibody or anti-mouse IgG antibody (Sigma Chemical Co.; dilution 1:200 to 1:50). Finally, the coverslips were extensively washed with DME, mounted on glass slides, and photographed using 10–20-s exposures for A431 cells and 60–120-s exposures for fibroblasts.

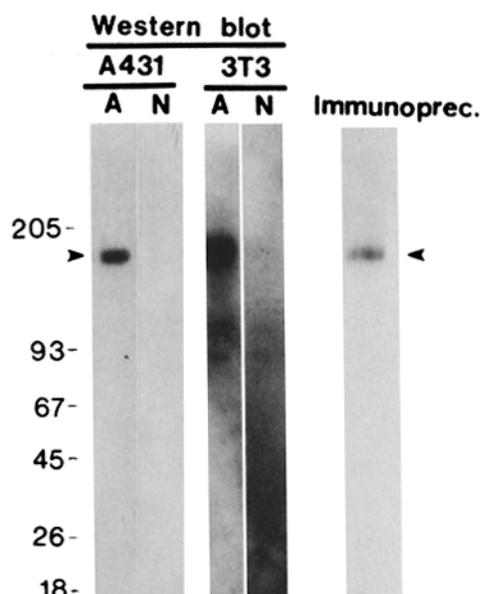
### <sup>125</sup>I-EGF Binding Assay

Cell monolayers, treated as described in Fig. 10 legend, were washed, then incubated at 4°C for 2 h with 20 nM <sup>125</sup>I-EGF (150,000 cpm/ng) in 1 ml of EBSS containing 20 mM Hepes, pH 7.4, and 1 mg/ml BSA (13, 14). After incubation the monolayers were washed free of unbound radioactivity, solubilized with 1 ml of 1 M NaOH, and counted in a gamma counter. Nonspecific binding was determined in the presence of 5  $\mu$ M unlabeled EGF.

## Results

### Antibody Specificity

Western blot analyses of crude membranes from A431 and 3T3 cells show that the rabbit anti-170-kD antibody binds specifically only to a 170-kD polypeptide (Fig. 1). The non-170-kD bands seen also bind to nonimmune IgG (Fig. 1). The 170-kD band co-migrates with the polypeptide that becomes covalently labeled with <sup>125</sup>I-EGF (Fig. 1). Similar immuno-



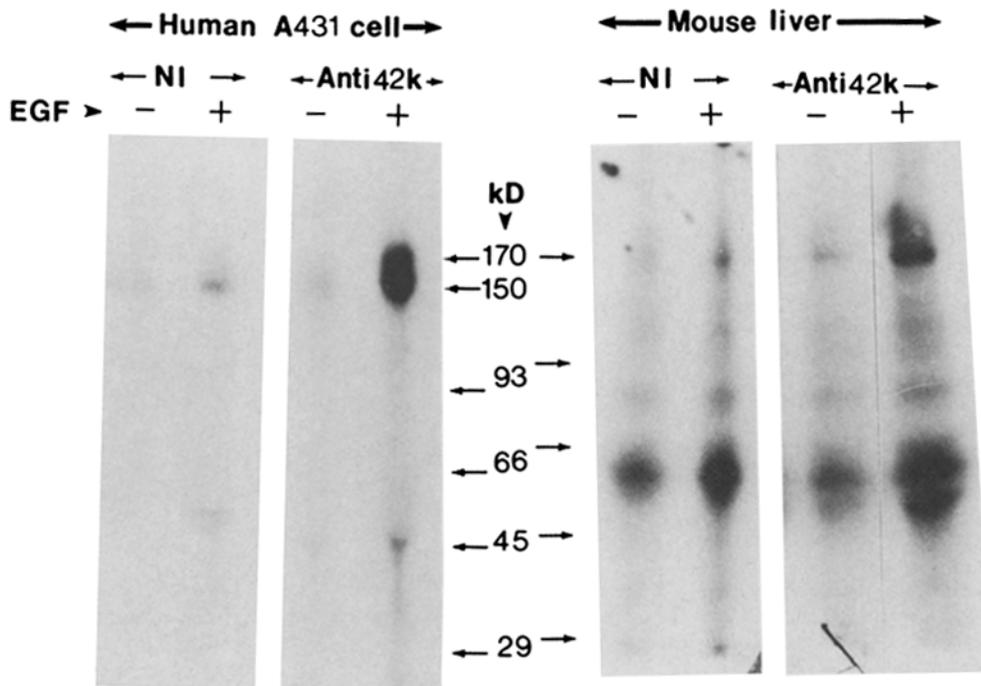
**Figure 1.** Antibody specificity depicted by Western blotting and immunoprecipitation analyses. A431 membranes (1  $\mu$ g protein) and 3T3 membranes (50  $\mu$ g protein) were subjected to SDS gel electrophoresis and Western blotting with anti-170-kD receptor rabbit antibody (A) or nonimmune rabbit IgG (N) as described in Materials and Methods. The autoradiographic exposure times for the Western blots with A431 and 3T3 cells were 15 h and 7 d, respectively. The immunoprecipitation experiments were conducted with 3T3 cell receptors covalently linked to <sup>125</sup>I-EGF using anti-170-kD receptor rabbit antibody as described in Materials and Methods. Identical experiments with 3T3 cells using nonimmune IgG or with NR-6 cells using antibody produced no immunoprecipitated protein at 170 kD or elsewhere.

blotting experiments with the anti-42-kD peptide antibody also revealed a high degree of specificity for the EGF receptor (not shown) and for the EGF receptor-derived 42-kD peptide (not shown) which was shown earlier to represent the tyrosine kinase domain of the receptor (2).

The specificity of the anti-42-kD peptide antiserum and the anti-170-kD receptor antiserum was further examined using Triton X-100 solubilized extracts of human A431 cell membranes and mouse hepatic membranes. As shown in Fig. 2 the anti-42-kD immune IgG immunoprecipitates a 170-kD protein (from both human and murine sources), which retains the capacity for EGF-stimulatable autophosphorylation in the isolated antibody-bound state. In A431 cells it also immunoprecipitates a 150-kD EGF receptor (Fig. 2), which is a proteolytic degradation product of the 170-kD receptor (2). A similar result was also obtained with the anti-170-kD receptor antibody (not shown).

### Domain Specificity of the Antibody Revealed by Indirect Immunofluorescent Staining of Cells

The anti-170-kD receptor antibody reacted well (at the plasma membrane) with permeabilized or nonpermeabilized human A431 cells (Fig. 3) and murine 3T3 cells (Fig. 4 B). No binding was seen with murine NR-6 cells (Fig. 4 C), an EGF-binding negative variant of 3T3 (13, 22). Thus the epitopes recognized by this antibody are EGF receptor-specific, and are present on the extracellular part of the receptor.



**Figure 2.** Immunoprecipitation of active EGF receptor kinase from detergent extracts of human A431 cells and mouse hepatic membranes by anti-42-kD peptide antibody and nonimmune (NI) rabbit IgG. Experimental procedures are described in Materials and Methods.

The anti-42-kD peptide antibody showed no reaction with nonpermeabilized human A431 cells (Fig. 3) or murine 3T3 cells (not shown). However permeabilization of the cells with methanol or with Triton X-100 revealed plasma membrane-localized reactions with the antibody in both A431 cells (Fig. 3) and 3T3 cells (Fig. 4 A). This indicates that the anti-42-kD peptide antibody is directed to receptor epitopes that are present exclusively in the cytoplasmic domain of the molecule.

The EGF receptor-negative mouse NR-6 cell line (13, 32) showed no reaction with the anti-42-kD antibody even in the permeabilized state (Fig. 4 C).

#### **Visualization of the Migratory Behavior of Receptor Kinase after EGF Treatment of Cells**

Migratory behavior of the receptor in murine 3T3 cells and human WI-38 cells was visualized by indirect immunofluorescent staining of permeabilized cells using the anti-42-kD peptide antibody and the anti-170-kD receptor antibody (Figs. 4 and 5). In experiments with the human WI-38 cell line, a murine antibody (15) directed to receptor extracellular domain was also used. In cells that were not treated with EGF, the receptor localized in the plasma membrane (Figs. 3-5). In cells that were treated with EGF at 37°C a perinuclear localization was seen, irrespective of the type of antibody used for staining (Figs. 4 and 5). The receptors in human A431 cells also showed a similar change in localization upon challenge with 40 nM EGF (not shown). The EGF-dependent change in receptor localization was not seen in cells that were treated with EGF at 4°C (not shown). The migratory process was unaffected in the presence of cycloheximide (not shown).

The presence of 10 mM methylamine enhanced the EGF-dependent perinuclear accumulation of receptors (Fig. 4 A).

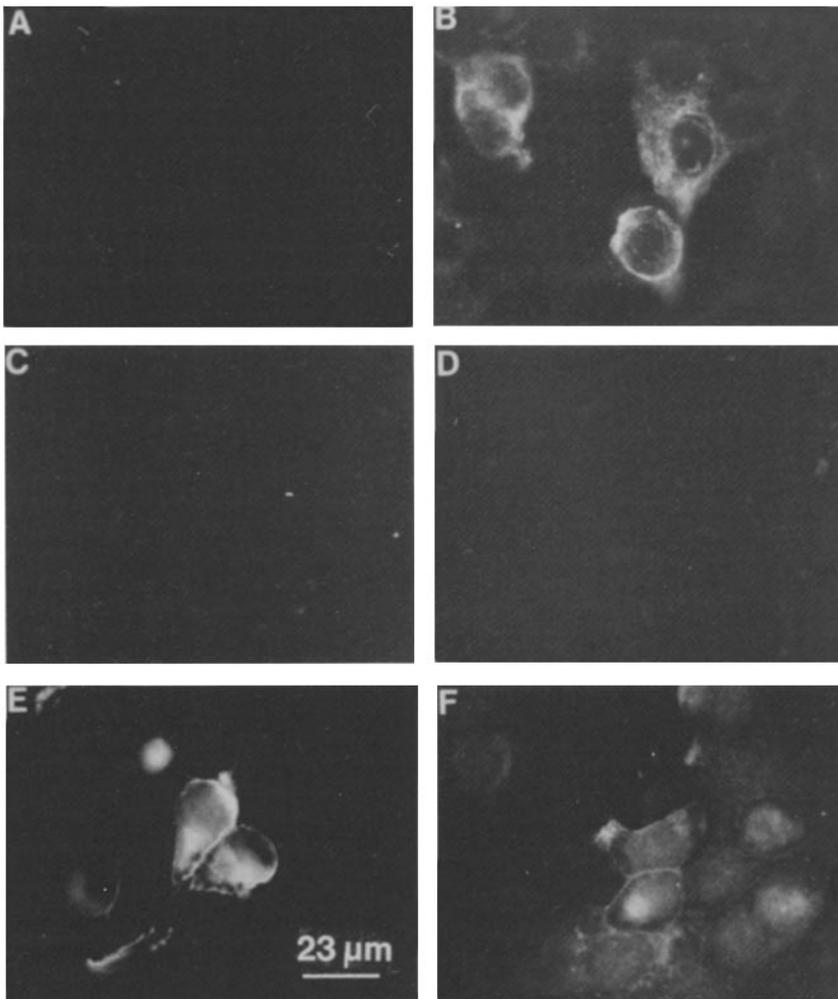
Chloroquine (100  $\mu$ M) also had a similar effect (not shown). However, these reagents alone, in the absence of EGF, did not affect receptor translocation to any significant extent.

#### **Effect of EGF Withdrawal on Receptor Location**

Cells treated with EGF at 37°C for 30 min showed no detectable EGF receptor-specific surface fluorescence in the nonpermeabilized state (Fig. 6 A). Permeabilization of these cells showed the presence of EGF receptors in the perinuclear region (Figs. 4-6). However, withdrawal of EGF from the medium and subsequent incubation at 37°C resulted in a rapid disappearance of these perinuclear receptors, accompanied by their appearance in the plasma membrane (Fig. 6). The process was seen irrespective of the type of antibody used for staining (Fig. 6, A and B), and was observed in both murine 3T3 cells (Fig. 6) and in human WI-38 cells (not shown). In double immunofluorescence experiments with WI-38 cells using rabbit anti-42-kD antibody and mouse anti-human receptor antibody, we found colocalization of fluorescein (anti-mouse) and rhodamine (anti-rabbit) fluorescence during EGF treatment and EGF withdrawal (not shown), suggesting that both domains of the receptor internalize and recycle in unison.

Translocation of receptors from the perinuclear region to the cell surface did not occur at 4°C (not shown). The presence of cycloheximide had no effect on this rapid phenomenon (Fig. 6 B), suggesting that the process involves recycling of receptors, rather than insertion of newly synthesized receptors into the plasma membrane. The migration was blocked in the presence of 10 mM methylamine (Fig. 6 B), 10  $\mu$ M monensin, and 100  $\mu$ M chloroquine (not shown).

The phenomenon was reexamined by  $^{125}$ I-EGF binding. Cells treated with unlabeled EGF (40 nM) at 37°C for 20 min showed no detectable  $^{125}$ I-EGF binding activity (Fig. 7). We



**Figure 3.** Indirect immunofluorescent staining of permeabilized and nonpermeabilized human A431 cells. (A, C, and E) Paraformaldehyde-fixed (nonpermeable) cells treated with anti-42-kD immune IgG (A), nonimmune IgG (C), anti-170-kD receptor immune IgG (E), and then stained with rhodamine-conjugated goat anti-rabbit antibody. (B, D, and F) Methanol-permeabilized cells treated with anti-42 kD immune IgG (B), nonimmune IgG (D), anti-170-kD receptor immune IgG (F), and then stained with rhodamine-conjugated goat anti-rabbit antibody.

tested whether incubation of these cells in EGF-free medium at 37°C would result in a return of the receptors to the surface. This was done by incubating the cells at 4°C for 2 h with a saturating concentration of  $^{125}\text{I}$ -EGF. In preliminary studies we found that cells treated at 4°C for 20 min with or without 40 nM EGF, then washed and incubated with 20 nM  $^{125}\text{I}$ -EGF at 4°C for 2 h, showed the same amount of radioactivity bound, irrespective of pretreatment or no treatment at 4°C with unlabeled EGF. Thus these binding conditions allow sufficient equilibration to occur (due to dissociation and reassociation) between cell-surface bound unlabeled EGF (<0.1 pmol) and the added excess (20 pmol) radiolabeled EGF, such that cell-bound radioactivity can be used for quantitation of surface receptor activity. Fig. 7 shows that when EGF-pretreated cells are incubated at 37°C in EGF-free medium, there is a progressive gain in  $^{125}\text{I}$ -EGF binding activity. This gain was blocked by methylamine (Fig. 7). In experiments where cells were incubated at 4°C after exposure to unlabeled EGF, there was no return of receptors to the cell surface (Fig. 7).

The results depicted in Figs. 6 and 7 indicate that internalized EGF receptors can rapidly recycle to the cell surface in a temperature-sensitive manner.

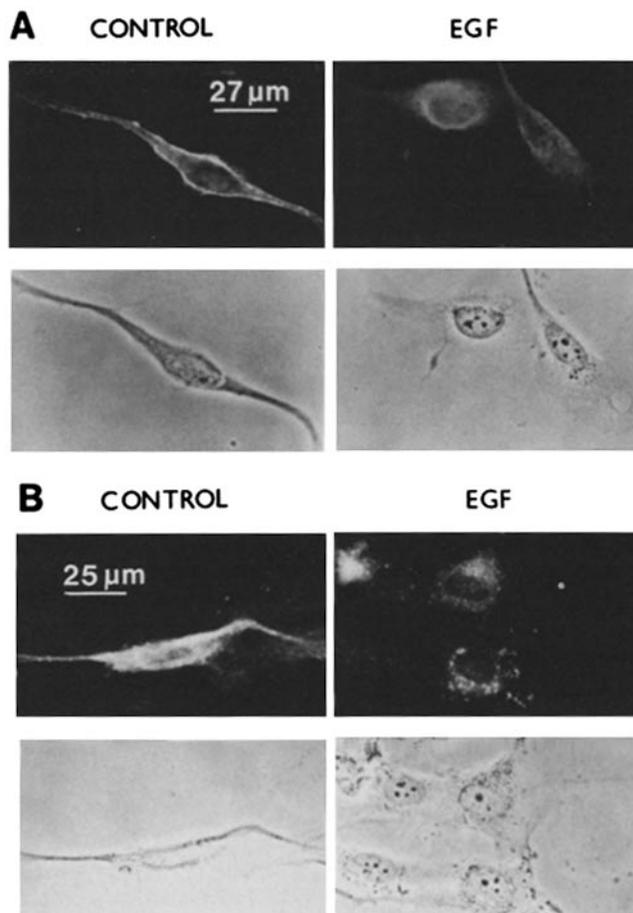
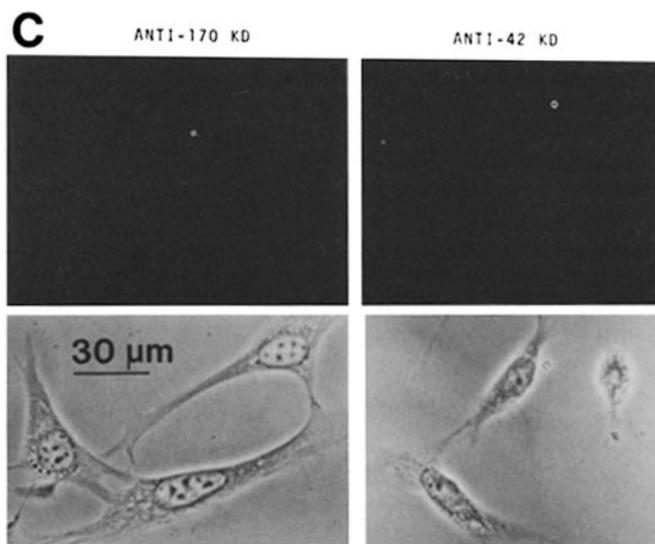
The extent of receptor recovery at the surface was drasti-

cally reduced when the period of pretreatment with EGF was 4 h instead of 30 min (not shown). Under such conditions, extensive receptor degradation ( $t_{1/2} \geq 1$  h) brings about a net decrease (i.e., down-regulation [1, 8]) in cellular EGF receptors (14, 16, 38), thus reducing the number of molecules that can recycle.

### Discussion

EGF receptor was the first growth factor receptor to be biochemically characterized. Subsequent studies on other growth factor receptors (platelet-derived growth factor receptor, insulin receptor, and insulin-like growth factor receptor) have revealed some unusual characteristics that mark them as belonging to a class quite different from those of other types of receptors. (a) Unlike the acetylcholine receptor and other ion-channel receptor polypeptides which span the plasma membrane more than once (12), the EGF and insulin receptor polypeptides appear to have only single transmembrane spanning segments (41, 42). (b) Unlike the ligand-accumulatory receptors (for low density lipoprotein, transferrin, asialoglycoproteins, etc.) which have very short cytoplasmically facing regions (50–70 residues long) (18, 28, 43), the growth factor receptors have large cytoplasmic do-





mains (500–700 residues long) (41, 42). (c) Unlike the  $\beta$ -adrenergic receptor, glucagon receptor, and other receptors which transmit signals through activation of a separable protein kinase, the growth factor receptors have an intrinsic ligand-activated kinase catalytic site built into the molecule (24, 26, 41, 42).

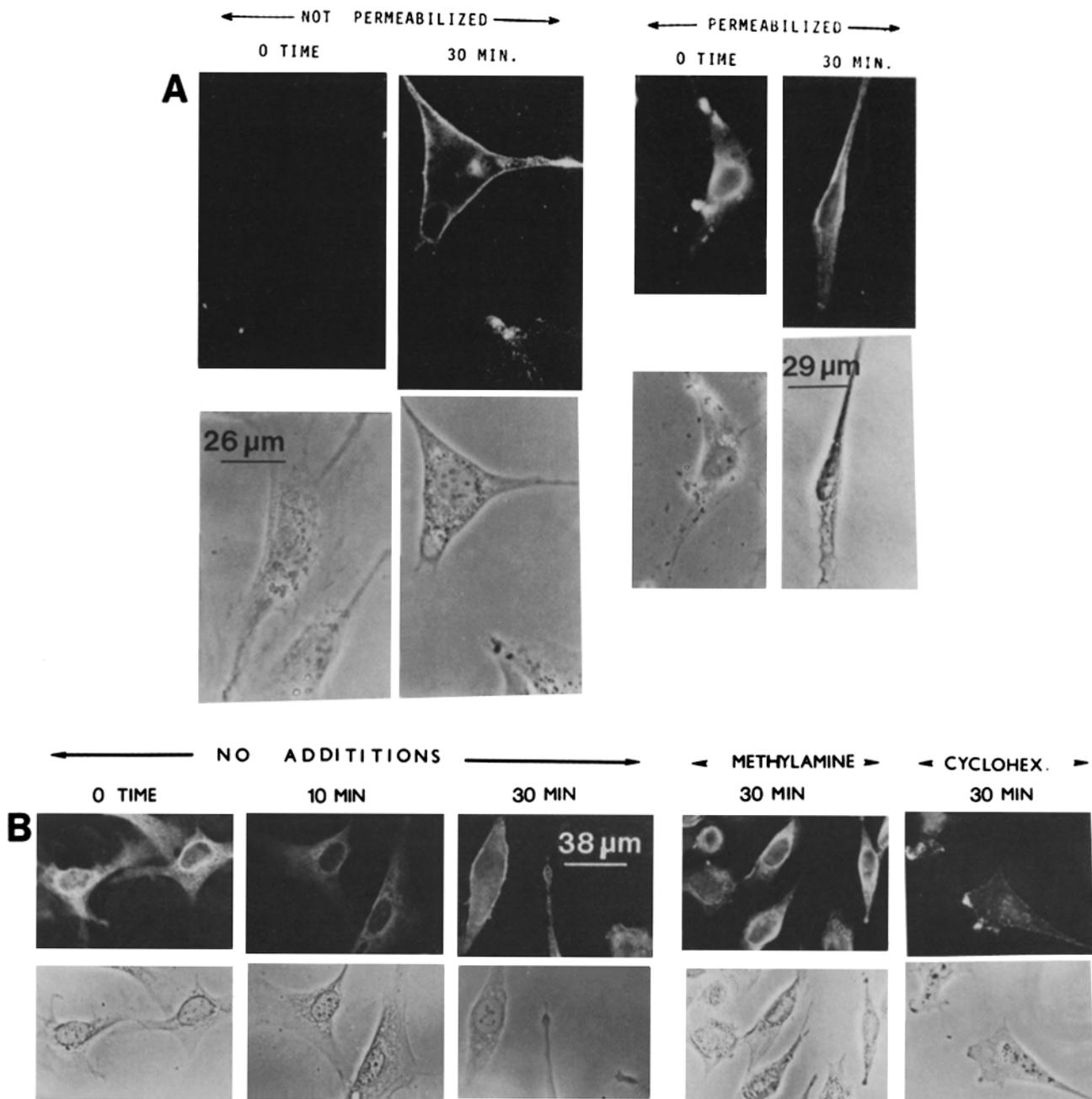
Thus growth factor receptors belong to a unique class of bipolar multi-sited proteins. The molecular and cellular aspects of how these membrane proteins induce growth stimulation remain as yet unknown.

As with many other ligand-receptor systems, EGF is known to be internalized after binding to its receptor. The degradative fate of the internalized receptor has been studied by covalent affinity labeling (14), and more recently by metabolic labeling using anti-receptor antibodies (4, 16, 38). Although the phenomenon has been known for quite some time, the precise location of the internalized receptor and the possibility of its recycling have been subjects of debate. In the present study we have examined location and migration of both occupied and unoccupied receptors by using anti-receptor antibodies whose specificity for the EGF receptor was established by various methods. The antibodies meet the following criteria: (a) they can specifically immunoprecipitate EGF receptors of both human and murine origin (Fig. 2); (b) the antibodies selectively bind to the EGF receptor in immunoblot experiments with A431 and 3T3 membranes (Fig. 1); (c) the antibodies do not bind to the NR-6 cell line which lacks EGF-receptors but which does contain other related growth factor receptors (Fig. 4 C).

One of the antibodies we have raised is directed to the kinase domain of the receptor. By using this antibody we have shown that the receptor kinase domain migrates to the perinuclear/nuclear envelope region in an EGF-dependent fashion. Studies with other anti-receptor antibodies indicate that both domains of the receptor are involved in migration. Inhibitors of protein biosynthesis have no effect on this process, suggesting that the appearance of immunoreactivity in the perinuclear region is not due to synthesis of new receptors in response to EGF, but rather is due to migration of pre-existing receptors from the plasma membrane to the perinuclear region.

**Figure 5.** EGF-induced migration of the EGF receptor to the perinuclear region of human WI-38 cells. (A) Indirect immunofluorescent staining with anti-42-kD peptide antibody; (B) staining with a murine anti-receptor antibody. See Materials and Methods and Fig. 4 legend for experimental details. The results of fluorescence and phase microscopy are shown in upper and lower panels, respectively.

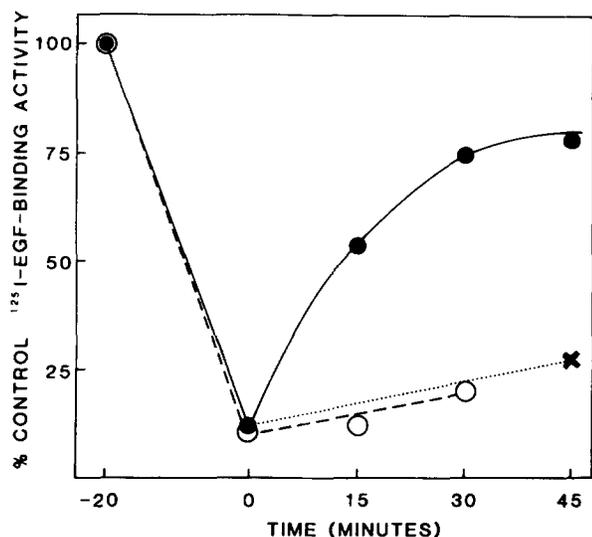
The results in Figs. 6 and 7 show that receptors, which accumulate in the perinuclear region in response to a brief treatment with EGF, rapidly return to the plasma membrane upon withdrawal of EGF. This contrasts with earlier findings on proteolytic degradation of internalized EGF receptors (4, 14, 16, 38), but supports earlier indirect evidence for EGF receptor recycling in hepatocytes (19). The data in Fig. 7 show that 70–75% of the receptors which had internalized (in response to 40 nM EGF) recycle to the surface after EGF withdrawal and that  $t_{1/2}$  for the recycling is <15 min. Assuming that all undegraded immunoreactive receptors can recycle, this would indicate that 25–30% of the receptors are degraded after a 30-min treatment with 40 nM EGF. This is consistent with the reported  $t_{1/2}$  of 1–1.5 h for receptor degradation at saturating concentrations of EGF. We have measured receptor recycling under conditions of EGF withdrawal, and the results suggest that all undegraded immunoreactive receptors rapidly recycle to the cell surface. The rapidity of this phenomenon suggests that in the presence of EGF a dynamic equilibrium between endocytic intake and recycling to surface determines the amount of receptors in the perinuclear region. An additional parameter is



**Figure 6.** Return of perineuronal receptors to the plasma membrane upon EGF withdrawal. (A) Indirect immunofluorescent staining of permeabilized and nonpermeabilized mouse 3T3 cells with anti-170-kD receptor antibody. (B) Indirect immunofluorescent staining of permeabilized 3T3 cells with anti-42-kD peptide antibody. Murine 3T3 cells were treated with 20 nM EGF at 37°C as described in Fig. 4 legend, then rapidly washed with DME-BSA and incubated at 37°C for the indicated times in EGF-free DME-BSA. In certain experiments, 10 mM methylamine or 20 μg/ml cycloheximide was included in the medium as indicated. Then the monolayers were fixed, left nonpermeabilized (as in A, left), or were permeabilized with Triton X-100 (A, right; B) and subjected to indirect immunofluorescent staining with antibody as described in Materials and Methods. The results of fluorescence and phase microscopy are shown in upper and lower panels, respectively. The two different scale bars in A represent different magnifications for nonpermeable and permeabilized cells.

provided by the irreversible step of receptor degradation (and of course receptor biosynthesis). These phenomena are EGF concentration-dependent, i.e., at sub-saturating (mitogenic) concentrations of EGF the dynamic equilibrium between surface-localized and internal receptors is likely to be more evident, and the  $t_{1/2}$  for receptor degradation is likely to be higher. Overall it appears that at a given time a major frac-

tion (~70%) of the internalized EGF receptors recycle to the plasma membrane while a minor population (<30%) undergoes degradation. The cumulative effect of this degradation, although not sizable after a brief incubation (as in Figs. 6 and 7), becomes appreciable after a 2 h (or greater) period of treatment of cells with saturating concentrations of EGF (14, 16, 38). The resultant down-regulation (1, 8) reduces the



**Figure 7.** Recycling of internalized EGF receptors. Washed monolayers of 3T3 cells in 35-mm dishes were incubated at 37°C for 30 min in DME with 1 mg/ml BSA containing either no addition (●, ×), or 10 mM methylamine (○). Then some dishes (-20 min point) were tested for <sup>125</sup>I-EGF binding activity as described in the last paragraph of Materials and Methods. Others were incubated at 37°C for 20 min in DME-BSA containing 40 nM EGF (unlabeled) without (●, ×) or with (○) 10 mM methylamine. After washing with DME-BSA, some dishes (0 time) were tested for <sup>125</sup>I-EGF binding activity. Others were kept at 37°C (●, ○) or 4°C (×) for the indicated times in 1 ml of DME-BSA without (●, ×) or with (○) 10 mM methylamine and then tested for <sup>125</sup>I-EGF binding activity. For each time point control <sup>125</sup>I-EGF binding was determined in cells that were treated identically as above except for the absence of EGF. 100% binding represents 180,000–200,000 cpm of specifically bound <sup>125</sup>I-EGF radioactivity. Nonspecific binding was ~10,000 cpm.

number of functional receptors that can return to the cell surface (see last paragraph or Results).

Direct evidence for recycling exists for the receptors for asialoglycoproteins, transferrin, and low density lipoprotein (3, 9, 22). It should be noted that these ligand accumulatory receptors are markedly different from the EGF receptor and other growth factor receptor kinases in the smallness and apparent nonfunctionality of their cytoplasmic domains (18, 28, 43). Thus the structural parameters that control migration and trafficking of ligand-accumulatory receptors may be quite different from those of receptor tyrosine kinases. In this context it is worth noting that indirect evidence for recycling has been reported for at least another receptor kinase, the insulin receptor (27), and that a dual subcellular location has been demonstrated for a related tyrosine kinase, the pp60<sup>c-src</sup> (33).

It is of interest to note that drugs such as methylamine (29) and monensin (3) inhibit the return of perinuclear receptors to the cell surface (Fig. 6). The state of activation of the receptor kinases trapped inside by these drugs will depend upon a variety of factors such as EGF occupancy and/or tyrosine phosphorylation. In any event, the normal process of receptor regeneration, involving return to the cell surface and re-occupancy by EGF, will be blocked by these drugs. If the continuing cycling of EGF receptors (from the inside to outside and back to inside) is needed for a critical intracel-

lular phosphorylation event or the amassing of a critical mitogenic signal, then any long-term interference with this process by drugs is likely to result in inhibition of receptor mitogenicity. On the other hand, a short-term incubation with these drugs may transiently increase the amount of active receptors in the perinuclear region and thereby have a slight activating effect.

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