MOLECULAR AND CELLULAR BIOLOGY, Aug. 1990, p. 4035–4044
0270-7306/90/084035-10$02.00/0

Vol. 10, No. 8

Evidence for Epidermal Growth Factor (EGF)-Induced Intermolecular Autophosphorylation of the EGF Receptors in Living Cells

A. M. HONEGGER,[1] A. SCHMIDT,[1] A. ULLRICH,[2] AND J. SCHLESSINGER[1]*

Rorer Biotechnology, Inc., 680 Allendale Road, King of Prussia, Pennsylvania 19406,[1] and Max-Planck-Institute fur Biochemie, 8033 Martinsried bei Munchen, Federal Republic of Germany[2]

Received 26 September 1989/Accepted 7 April 1990

In response to epidermal growth factor (EGF) stimulation, the intrinsic protein tyrosine kinase of EGF receptor is activated, leading to tyrosine phosphorylation of several cellular substrate proteins, including the EGF receptor molecule itself. To test the mechanism of EGF receptor autophosphorylation in living cells, we established transfected cell lines coexpressing a kinase-negative point mutant of EGF receptor (K721A) with an active EGF receptor mutant lacking 63 amino acids from its carboxy terminus. The addition of EGF to these cells caused tyrosine phosphorylation of the kinase-negative mutant by the active receptor molecule, demonstrating EGF receptor cross-phosphorylation in living cells. After internalization the kinase-negative mutant and CD63 have separate trafficking pathways. This limits their association and the extent of cross-phosphorylation of K721A by CD63. The coexpression of the kinase-negative mutant together with active EGF receptors in the same cells suppressed the mitogenic response toward EGF as compared with that in cells that express active receptors alone. The presence of the kinase-negative mutant functions as a negative dominant mutation suppressing the response of active EGF receptors, probably by interfering with EGF-induced signal transduction. It appears, therefore, that crucial events of signal transduction occur before K721A and active EGF receptors are separated by their different endocytic itineraries.

The receptor (EGFR) for epidermal growth factor (EGF) is a member of the tyrosine kinase family of growth factor receptors, which regulate cell proliferation, differentiation and oncogenesis (reviewed in references 7, 32, 33, and 41). The binding of EGF to the EGFR stimulates the cytoplasmic kinase activity, resulting in tyrosine phosphorylation of the receptor itself and of exogenous substrates (reviewed in references 14, 33, and 41). The protein tyrosine kinase activity of EGFR is essential for signal transduction; kinase-negative EGFR mutants fail to stimulate any EGF-induced cellular responses (3, 9, 12, 20, 21, 27).

Four autophosphorylation sites have been identified in the carboxy terminus of EGFR (5, 22, 36). Additional minor sites are exposed when the main autophosphorylation sites are deleted (22). The binding of EGF to the EGFR causes rapid receptor oligomerization both in vitro and in intact cells (4, 32–34, 40, 42). Receptor oligomerization plays an important role in the mechanism underlying EGF-induced activation of the intrinsic kinase activity (32, 33). However, the kinase activity of EGFR can also be regulated by ligand-independent mechanisms that do not involve receptor oligomerization (16, 28). The fact that ligand-induced receptor activation is mediated by receptor oligomerization complicates the interpretation of classical kinetic experiments for the analysis of the mechanism of receptor autophosphorylation. This is because it is impossible to dissociate the activation step from the process of receptor autophosphorylation. Using an alternative approach, we were able to show that in vitro a kinase-negative point mutant (K721A) was phosphorylated by the active kinase of a second mutant EGFR in a ligand-dependent manner (11). This intermolecular phosphorylation was indistinguishable from the autophosphorylation of the wild-type (wt) receptor, indicating that all substrate sites are accessible to intermolecular phosphorylation. Although these results do not exclude an intramolecular contribution for the mechanism of receptor autophosphorylation, they strongly suggest that autophosphorylation is mediated exclusively by cross-phosphorylation. It is unlikely that an intramolecular and an intermolecular mechanism will both phosphorylate the same sites with similar stoichiometry and ligand dependence.

In a living cell, EGFR is subjected to more stringent constraints on conformation and orientation. Its freedom of movement is restricted to lateral and rotational mobilities in the plane of the plasma membrane (33, 34, 42), which also retains the receptors in a nearly parallel orientation. Moreover, the reduced dimensionality of the lipid bilayer increases the local concentration of the receptor, thus increasing the efficiency of receptor-receptor interactions (33).

To address the mechanism of receptor autophosphorylation in living cells, we generated NIH 3T3 cells, lacking endogenous EGFR, that coexpress on their cell surface a kinase-negative EGFR mutant (K721A) and a mutant receptor with active kinase (9–13). Here we show that EGF induces the phosphorylation of the kinase-negative mutant by the active EGFR. This demonstrates that autophosphorylation in living cells can proceed by an intermolecular mechanism. Moreover, the presence of the kinase-negative mutant reduced the mitogenic response to subsaturating concentrations of EGF as compared with that in cells expressing only the active receptor mutant. The defective receptor mutant functions as negative dominant mutation for biological responsiveness, probably by interfering with EGF-induced signal transduction.

* Corresponding author.

MATERIALS AND METHODS

Transfection. NIH 3T3 cells (clone 2.2) lacking endogenous EGF receptors (9, 12) were grown in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal calf serum (FCS). Cultured cells growing in 10-cm dishes were transfected with 10 to 20 μg of plasmid DNA per dish by the calcium phosphate precipitation technique (39). The plasmid contained dihydrofolate reductase and the neomycin resistance genes as selectable markers. Geneticin (G418, 0.9 μg/ml; GIBCO) was added to the medium to select for neomycin-resistant transfectants. Clones were picked after 3 weeks and characterized as previously described (9, 12, 18, 20, 21). The cell line designated K721A (clone 19), expressing approximately 300,000 kinase-negative receptors per cell, was used as parental cell line for a second transfection with 10 to 20 μg of DNA per 10-cm dish of expression plasmid containing a 63-amino-acid deletion mutant of the EGF receptor designated CD63. As a control, the same construct was also transfected into parental NIH 3T3 (clone 2.2) cells. Plasmid pSVgpt was cotransfected at a concentration 10 times lower than that of the plasmid encoding the receptor mutant to provide guanosine phosphorybosyl transferase as a selectable marker. The transfected cells were grown in a selection medium made up of DMEM, 10% calf serum, 1% penicillin-streptomycin, 1% glutamine, 40 μg of mycophenolic acid per ml, 5 μM aminopterine, 10 μg of thymidine per ml, 100 μM hypoxanthine, 250 μg of xanthine per ml, and 10 μg of glycine per ml. Individual colonies were isolated, and the clones were characterized. In some lines that did not initially yield sufficiently high expression, coamplification of both constructs was achieved by selection with gradually increasing doses of methotrexate (initial concentration, 50 nM; final concentration, 1.6 μM).

$^{32}P_i$ labeling and receptor phosphorylation in intact cells. Cells expressing wt EGFR (HER cells [9, 12]), a mutant with a C-terminal truncation of 63 amino acids (CD63) and lacking two major autophosphorylation sites (20), and a point mutant in which lysine 721 has been replaced by alanine (K721A), leading to the complete loss of kinase activity in vitro and in living cells (9, 12, 27), were plated on fibronectin-coated 15-cm dishes and allowed to grow to confluence. The cells were washed three times with phosphate-free RPMI 1640 and incubated overnight in 10 ml of phosphate-free RPMI 1640 containing 0.5% dialyzed FCS, 10 μM Na_3VO_4 to increase tyrosine phosphorylation, and 2 mCi of $^{32}P_i$ (Dupont, NEN Research Products, Boston, Mass.) per plate. The cells were incubated for 30 min at 37°C with 100 nM phorbol-12-myristate-13-acetate (PMA) or for 15 min at 37°C with 500 ng of EGF per ml. They were washed extensively with phosphate-buffered saline (PBS) containing 10 μM sodium orthovanadate and lysed in 1 ml of lysis buffer containing a mixture of protease and phosphatase inhibitors (lysis buffer contained 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] [pH 7.5], 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, and 1% Triton X-100; protease inhibitors were 4 μg of phenylmethylsulfonyl fluoride per ml, 10 μg of leupeptin per ml, and 10 μg of aprotinin per ml; phosphatase inhibitors were 100 mM NaF, 30 mM *p*-nitrophenyl phosphate, 10 mM sodium pyrophosphate, and 200 μM sodium orthovanadate).

Immunoprecipitation. Protein A-Sepharose (30 μg; Sigma Chemical Co., St. Louis, Mo.) was washed with 20 mM HEPES and incubated for 30 min at room temperature with 10 μl of different anti-EGFR antibodies, including MAb108 (a monoclonal antibody against the extracellular portion of the EGFR) and anti-C (anti-peptide antiserum against the carboxy-terminal peptide of EGFR). RK2 and anti-F are two antipeptide antisera that recognize the cytoplasmic domain of EGFR (17–20). We also used an antiserum specific for synthetic peptide derived from phospholipase-Cγ (23, 26, 37) (anti-PLC). The various solubilized cell samples were incubated with the immobilized antibodies for 1 h at 4°C, centrifuged, and washed four times with high-salt HNTG (20 mM HEPES [pH 7.5], 500 mM NaCl, 0.1% Triton X-100, 10% glycerol) and once with HNTG containing 150 mM NaCl. Finally, 50 μl of sodium dodecyl sulfate (SDS) sample buffer was added, and the sample was heated to 95°C for 4 min. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 7% polyacrylamide gel and detected by autoradiography of the dried gel.

Phosphoamino acid analysis and phosphopeptide mapping. Radioactive bands were cut from the gel, and the gel slices were incubated overnight under gentle shaking with 10% methanol to rehydrate the gel and to extract the SDS. The gel was lyophilized, and 20 μl of a 0.1-μg/μl solution of trypsin (sequencing grade; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 50 mM NH_4HCO_3 (pH 7.0) added to each slice. The gel pieces were incubated in 0.5 ml of 50 mM NH_4HCO_3 (pH 7.0) for 12 h at 37°C with shaking. The supernatant was collected, the gel pieces were washed twice with 0.25 ml of buffer, and the collected supernatants were dried in a Speedvac rotatory evaporator and dissolved in 100 μl of 0.1% trifluoroacetic acid.

A portion of each sample (2,000 Cherenkov cpm) was dried in a hydrolysis tube and hydrolyzed in 6 N HCl for 2 h at 110°C under vacuum. The hydrolysate was dried well to remove excess acid and then dissolved in 10 μl of a solution containing 0.6 mg each of phosphotyrosine, phosphothreonine, and phosphoserine standards per ml (13). The samples were spotted onto 10- by 10-cm glass-backed cellulose thin-layer chromatography plates. In the first dimension, the phosphoamino acids were separated by electrophoresis in acetic acid-formic acid-water (78:25:897, vol/vol/vol; pH 1.9) for 30 min at 1,500 V. The plates were dried, and the second dimension was developed by electrophoresis in acetic acid-pyridine-water (50:5:945, vol/vol/vol; pH 3.5) for 25 min at 1,000 V. The plates were dried and stained with ninhydrin (0.3% in acetone) for visualization of phosphoamino acid standards, whereas the radioactive phosphoamino acids were detected by autoradiography of the thin-layer chromatography plates. ^{32}P-labeled peptide samples were analyzed by reversed-phase high-pressure liquid chromatography on a C_{18} column with a 40-min gradient from 0 to 40% acetonitrile in 0.1% trifluoroacetic acid in water at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and counted in a β-counter to determine the elution profile. Peak fractions of sufficient activity were dried and hydrolyzed, and their phosphoamino acid composition was determined as described above.

In vitro phosphorylation. Confluent cells were lysed in lysis buffer without phosphatase inhibitors and immunoprecipitated with either MAb108 or anti-C antibodies against EGFR as described above. The immunoprecipitates were incubated with 0.5 μCi of [γ-^{32}P]ATP per sample, 5 μM unlabeled ATP, and 5 mM $MnCl_2$ for 15 min on ice. The samples were analyzed by SDS-PAGE, the radioactive bands were excised and digested with trypsin, and the eluted phosphopeptides were mapped by reversed-phase high-pressure liquid chromatography as described above.

Analysis of tyrosine phosphorylation in intact cells with

anti-phosphotyrosine antibodies. Cells were grown to confluence in fibronectin-coated 15-cm dishes and starved for 12 h in DMEM containing 0.5% FCS and 10 μM sodium orthovanadate. The cells were either lysed directly or exposed for 10 min at 37°C to 500 ng of EGF per ml before solubilization in lysis buffer containing phosphatase inhibitors. Half of each lysate was immunoprecipitated with MAb108 bound to protein A-Sepharose, and the second half was immunoprecipitated with anti-C bound to protein A-Sepharose. Lysates were incubated with the beads for 90 min at 4°C, and then the immunoprecipitates were washed four times with HNTG containing 500 mM NaCl and once with low-salt (150 mM NaCl) HNTG solution. Each immunoprecipitate was split into three samples. Two were mixed with SDS sample buffer, heated to 95°C, analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either an anti-peptide antiserum against EGFR (anti-F) or with anti-phosphotyrosine antiserum. Subsequently the blots were labeled with ^{125}I-labeled protein A. The third sample was autophosphorylated in vitro and analyzed by SDS-PAGE and autoradiography.

Covalent cross-linking experiments. Cells were grown to confluence in fibronectin-coated 10-cm dishes. The confluent cell layers were washed three times with PBS and incubated for 15 min at 37°C or for 60 min at 20°C in PBS with 500 ng of EGF per ml in the presence of 15 mM of the covalent cross-linking agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (4). The cells were washed and incubated for 5 min at 20°C with PBS containing 150 mM glycine to block unreacted cross-linker, washed again with PBS, lysed, immunoprecipitated, and analyzed by SDS-PAGE on a 6% polyacrylamide gel. After transfer to nitrocellulose, receptor monomers and dimers were visualized by immunoblotting with anti-EGFR anti-peptide antibodies (anti-F).

[^{3}H]thymidine incorporation experiments. Cells were plated in fibronectin-coated 24 well Costar dishes at a density of 20,000 cells per well. After 2 days, the medium was changed to DMEM containing 0.5% FCS and 25 mM HEPES (pH 7.4). The cells were starved for 2 or 3 days with daily changes of the starvation medium. Different concentrations of EGF were added; 18 h later, 0.5 μCi of [^{3}H] thymidine per well was added, and the cells were allowed to grow for 4 h. The cells were then washed three times with PBS, 0.5 ml of ice-cold trifluoroacetic acid was added to each well, and the cells were incubated for 30 min on ice. The precipitate was washed two times with PBS and lysed by incubation with 0.5 ml of 0.1 N NaOH for 30 min at 30°C. The lysate was neutralized, and the amount of [^{3}H]thymidine incorporated into DNA was determined by scintillation counting in a β-counter.

RESULTS

The mechanism of EGFR autophosphorylation in living cells was studied with transfected NIH 3T3 cells (clone 2.2, which lacks endogenous EGFR) expressing different EGFR mutants. HER14 cells are NIH 3T3 cells expressing approximately 300,000 wt EGFR molecules per cell. CD63 cells are NIH 3T3 cells expressing approximately 300,000 molecules per cell of an active EGFR mutant lacking 63 amino acids from the carboxy terminus and therefore devoid of two tyrosine autophosphorylation sites (8, 10, 20). K721A cells express approximately 300,000 molecules per cell of kinase-negative point mutant. K721A/CD63 cell lines were established by transfecting the CD63 containing the construct into K721A cells and selecting cell lines that express different amounts of CD63 against a constant background of K721A (Honegger et al., in press).

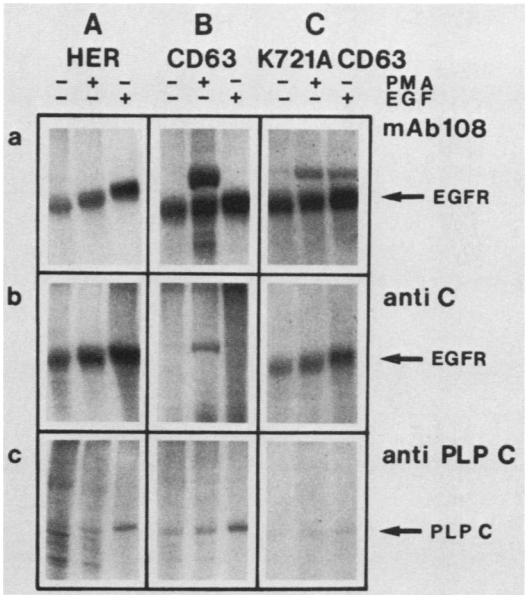

FIG. 1. Autophosphorylation and EGF-induced phosphorylation of phospholipase Cγ in intact cells labeled with $^{32}P_i$. Cells expressing wt EGFR (HER; panel A) or C-terminally truncated active receptor (CD63; panel B) or coexpressing the kinase-negative receptor and CD63 (K721A/CD63; panel C) were labeled overnight with $^{32}P_i$ in phosphate-free medium. After simulation with either PMA (100 nM, 30 min at 30°C) or EGF (500 ng/ml, 15 min at 37°C), the cells were lysed in the presence of phosphatase inhibitors. The cell lysates were immunoprecipitated with either MAb108 to isolate all forms of the EGFR (HER, CD63, and K721A; row a), with anti-C to isolate only full length receptor (HER and K721A; row b), or with anti-PLC to isolate phospholipase Cγ (row c). After analysis by SDS-PAGE and autoradiography, the receptor bands were excised from the gel. Phosphoamino acid composition (Fig. 2) and phosphopeptide maps (Fig. 3) of these samples were also determined.

Several approaches were used to determine whether the binding of EGF to the kinase-active receptor induces the phosphorylation of the kinase-negative mutant expressed in the same cells.

Experiments with intact cells labeled with $^{32}P_i$. The various cells were labeled overnight with $^{32}P_i$ and then treated with either 500 ng of EGF per ml for 15 min at 37°C or with 100 nM PMA for 30 min at 37°C. After solubilization, EGFR was immunoprecipitated with different antibodies. MAb108, which recognizes the extracellular domain of EGFR, was able to immunoprecipitate all mutant EGFRs described in this report (Fig. 1, row a). Anti-C antibodies are directed against a synthetic peptide from the carboxy-terminal sequence of EGFR and therefore immunoprecipitated wt receptors and kinase-negative mutant but not the CD63 deletion mutant, because it lacks 63 carboxy-terminal amino acid residues (Fig. 1, row b). We have previously shown by using anti-C antibodies for immunoprecipitation that EGF induces association between CD63 and K721A. This experiment revealed the existence of hetero-oligomers of CD63 and K721A and demonstrated EGF-induced cross-phosphorylation of K721A by CD63 (11; Honegger et al., in press). To be able to distinguish K721A phosphorylation from CD63 phosphorylation, we washed anti-C immunoprecipitates extensively with high-salt buffer. No CD63 was immunoprecipitated from CD63 cells under these conditions (Fig. 1, row b).

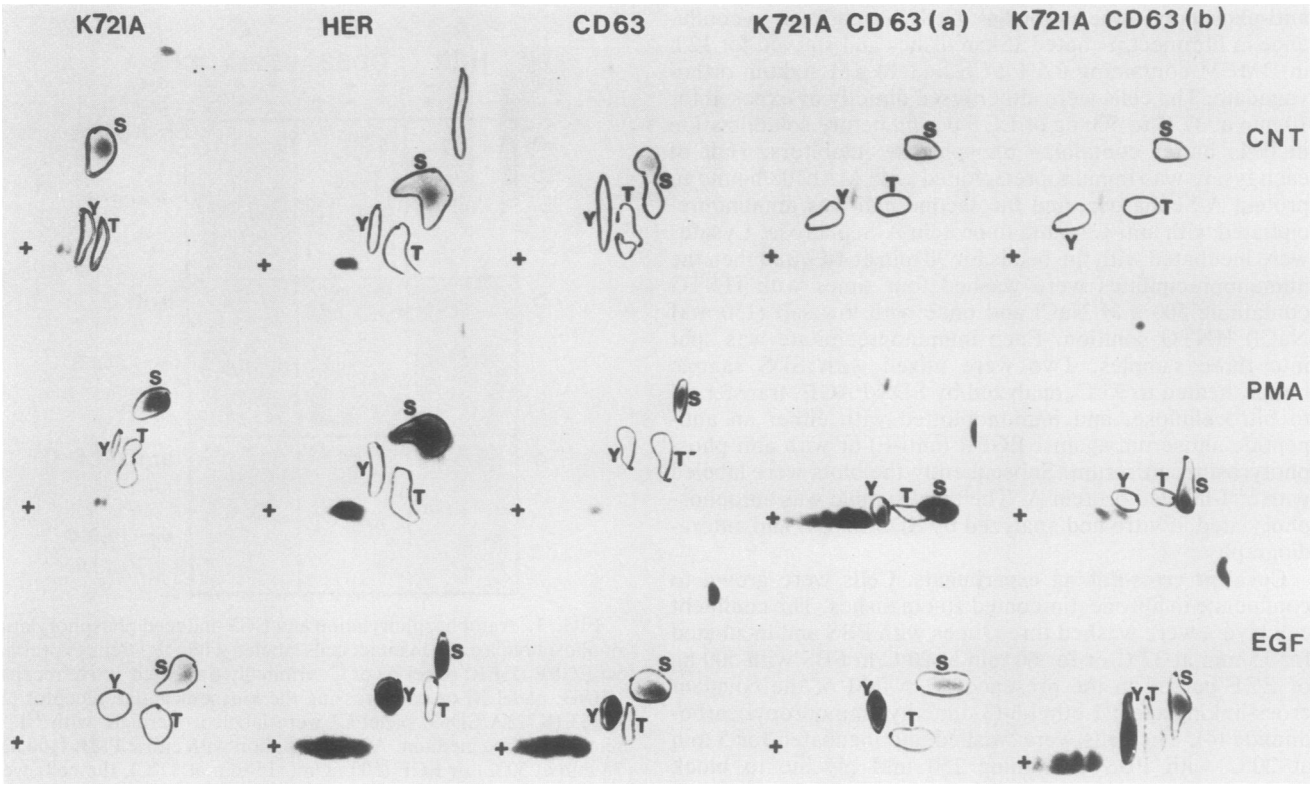

FIG. 2. Phosphoamino acid analysis of EGFR phosphorylated in intact $^{32}P_i$-labeled cells. NIH 3T3 cells expressing wt receptor (HER), K721A, CD63, and both K721A and CD63 (K721A/CD63 cells) were immunoprecipitated from either PMA or EGF-stimulated, $^{32}P_i$-labeled cells with either MAb108 or anti-C antibodies (18). After analysis by SDS-PAGE, the receptor bands were excised and digested with trypsin. The eluted tryptic peptides were hydrolyzed, and their phosphoamino acid composition was determined by two-dimensional electrophoresis (13).

We have previously shown that basal phosphorylation of these three receptor species occurs mainly on serine and threonine residues (9, 12). PMA treatment enhanced mainly serine phosphorylation and, to a lesser extent, threonine phosphorylation. EGF treatment led to a marked increase of tyrosine phosphorylation. An additional phosphoprotein of approximately 200,000 kilodaltons was immunoprecipitated from cells expressing the CD63 mutant. This protein was phosphorylated in response to PMA and was almost exclusively phosphorylated on serine residues. The phosphopeptide map of this protein was unrelated to the phosphopeptide map of EGFR (data not shown).

After autoradiography, the radioactive EGFR bands (Fig. 1) were excised from the gels and digested with trypsin. The receptor fragments were eluted; a portion of each sample was hydrolyzed, and its phosphoamino acid composition was determined by two-dimensional electrophoresis (Fig. 2) as described previously (13). In quiescent cells, basal phosphorylation was due to phosphoserine and threonine, whereas phosphotyrosine was essentially undetectable. PMA treatment predominantly increased serine and, to a lesser extent, threonine phosphorylation. The kinase-negative mutant K721A was immunoprecipitated from K721A/CD63 cells with antibodies against a synthetic peptide from the carboxy terminus (anti-C). Phosphorylation of the kinase-negative mutant was clearly enhanced by EGF. The immunoprecipitation experiment with anti-C was performed under conditions that prevent coimmunoprecipitation of CD63 receptor with the K721A mutant. K721A expressed alone never showed detectable tyrosine phosphorylation; hence, tyrosine phosphorylation of K721A in response to EGF in these cells is due to cross-phosphorylation of K721A by CD63.

The phosphopeptide maps of the various receptor mutants were compared. Tryptic digests of phosphorylated EGFR were separated on a C_{18} reversed-phase high-pressure liquid chromatography column with an acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions were collected, and radiolabeled peptides were detected in a β-counter. The phosphoamino acid content of peak fractions was determined (13). The phosphopeptide maps of in vitro phosphorylated wt and the C-terminally truncated mutant CD63 showed clear differences. The CD63 mutant lacks autophosphorylation site tyrosines in positions 1173 and 1148, and thus lacked peaks a and c from the phosphopeptide map (Fig. 3A). When a mixture of K721A and CD63 mutant receptors was incubated with [γ-^{32}P]ATP, a phosphopeptide map containing peptides a and c was observed, confirming our earlier results that, in vitro, CD63 was able to phosphorylate the kinase-negative K721A mutant (11).

A similar result was obtained when the phosphopeptide maps of EGFR phosphorylated in intact cells were analyzed and compared (Fig. 3B). The phosphopeptide map of EGFR isolated from intact cells was more complex due to the presence of additional serine and threonine phosphorylated peptides. Six tyrosine phosphorylated peaks appeared in the positions corresponding to those seen in the in vitro phosphorylated wt EGFR. The phosphopeptide map of the CD63 mutant lacked the peaks corresponding to tyrosines 1173 and 1148 (peaks c and a, respectively), whereas these peaks were

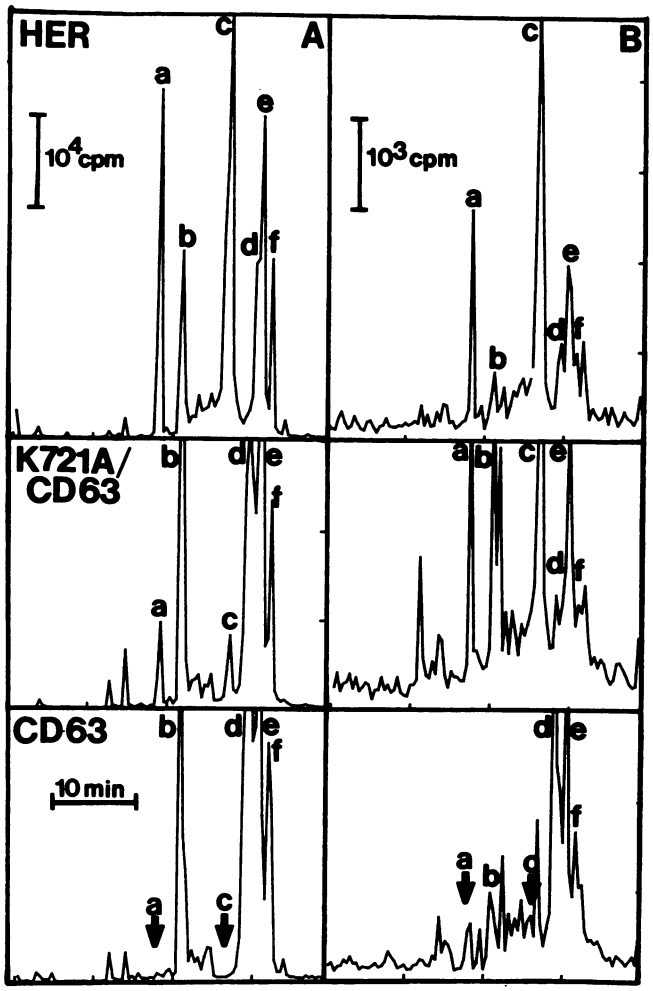

FIG. 3. Tryptic phosphopeptide maps of EGFR phosphorylated in vitro with [γ-^{32}P]ATP and in intact $^{32}P_i$-labeled cells. NIH 3T3 cells expressing wt EGFR (HER), C-terminally truncated receptor coexpressed with kinase-negative receptor (K721A/CD63), and CD63 alone were allowed to phosphorylate in vitro in the presence of [γ-^{32}P]ATP after immuniprecipitation with MAb108 (A) or immunoprecipitated from EGF-stimulated $^{32}P_i$-labeled cells (B). After analysis by SDS-PAGE, the receptor bands were excised and digested with trypsin. The eluted tryptic peptides were separated by reversed-phase high-pressure liquid chromatography with a 0 to 30% acetonitrile gradient in 0.1% trifluoroacetic acid to elute the peptides from an rp18 column at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and counted, and active fractions were hydrolyzed for phosphoamino acid analysis.

present in the phosphopeptide map obtained from cells coexpressing K721A and CD63, indicating that the kinase-negative mutant K721A was indeed phosphorylated by CD63 in living cells. Additional peaks either were below the detection limit of phosphoamino acid analysis or contained predominantly phosphothreonine and/or phosphoserine residues.

Detection of tyrosine phosphorylated EGF receptor by immunoblotting with anti-phosphotyrosine antibodies. Cells expressing the wt, CD63, and two different lines of K721A/CD63 were grown to confluence in fibronectin-coated 15-cm dishes. The cells were starved overnight in DMEM containing 0.5% FCS, 25 mM HEPES, and 10 μM sodium orthovanadate. The cells were stimulated for 5 min with 500 ng of EGF per ml, washed, and lysed in the presence of phosphatase inhibitors. Each lysate was divided into two samples; one was immunoprecipitated with MAb108, which recognizes the wt, CD63, and K721A, and the second was immunoprecipitated with anti-C, which recognizes the wt and K721A but not the CD63 mutant. Each immunoprecipitate was further divided into three samples, which were subjected to SDS-PAGE analysis and immunoblotted with three different antibodies as follows: (i) anti-F antibodies, an anti-EGFR antipeptide antiserum directed against a peptide derived from the juxtamembrane region of EGFR, thus monitoring the total amount of receptor protein in the various samples (Fig. 4A); (ii) anti-C antibodies, which recognize the wt and K721A receptors but not the CD63 mutant receptor; and (iii) anti-phosphotyrosine antibodies to detect endogenous phosphate incorporated into tyrosine residues of the different receptor species during in vivo phosphorylation (Fig. 4C). In vitro autophosphorylation experiments with [γ-^{32}P]ATP were also performed to detect kinase activity of CD63 and wt EGF in the different immunoprecipitates.

Comparable amounts of total EGFR proteins were produced by all four cell lines (Fig. 4A). Tyrosine phosphorylated EGFR from EGF-treated cells migrated more slowly in SDS gels (13, 23). CD63 lacks two autophosphorylation sites, and therefore its electrophoretic mobility was less affected by autophosphorylation. Samples containing a mixture of CD63 and K721A showed more retardation in electrophoretic mobility than CD63 alone, already suggesting that K721A may be phosphorylated in response to EGF in these cells. Figure 4A, row b, depicts autoradiograms of the same samples shown in Fig. 4A, row a, but which were immunoprecipitated with anti-C antibodies. The wt receptor was precipitated by anti-C with an efficiency similar to that with MAb108. As anticipated, CD63 was not recognized by this antiserum. The two cell lines coexpressing K721A and CD63 expressed different amounts of the two receptor species. Tyrosine phosphorylation of K721A was clearly detected by the electrophoretic shift in the immunoblots of EGFR presented in Fig. 4A, row b. However, longer exposure was required for the detection of tyrosine phosphorylation of K721A expressed in the second cell line, which expressed fewer CD63 molecules.

To detect the presence of active EGFR kinase in these cell lines, a sample of each immunoprecipitate was incubated with [γ-^{32}P]ATP and analyzed by SDS-PAGE and autoradiography (Fig. 4B). Since the receptor was already phosphorylated by the endogenous ATP pool, samples from EGF-treated cells incorporated less [^{32}P]phosphate than samples from unstimulated cells. All receptor samples immunoprecipitated with MAb108 (which recognizes the wt, CD63, and K721A) contained active EGFR kinase. Anti-C antibodies immunoprecipitated active kinase only from cells expressing wt receptors. These results show that the washing conditions used prevented coimmunoprecipitation of CD63 and K721A mutants. Immunoprecipitates with anti-C antibodies from K721A/CD63 cells clearly contained only K721A; therefore the phosphorylation detected in these samples is due to cross-phosphorylation of K721A by CD63.

Immunoblotting analyses with anti-phosphotyrosine antiserum (Fig. 4C and D) indicated that basal tyrosine phosphorylation of EGFR was undetectable, whereas a strong signal was obtained in response to EGF. Cross-phosphorylation of K721A by CD63 was clearly detectable. Pretreatment of the cells with 10 μM phenylarsine oxide (PAO) for 15 min at 37°C before the addition of EGF significantly enhanced both basal and EGF-stimulated autophosphoryla-

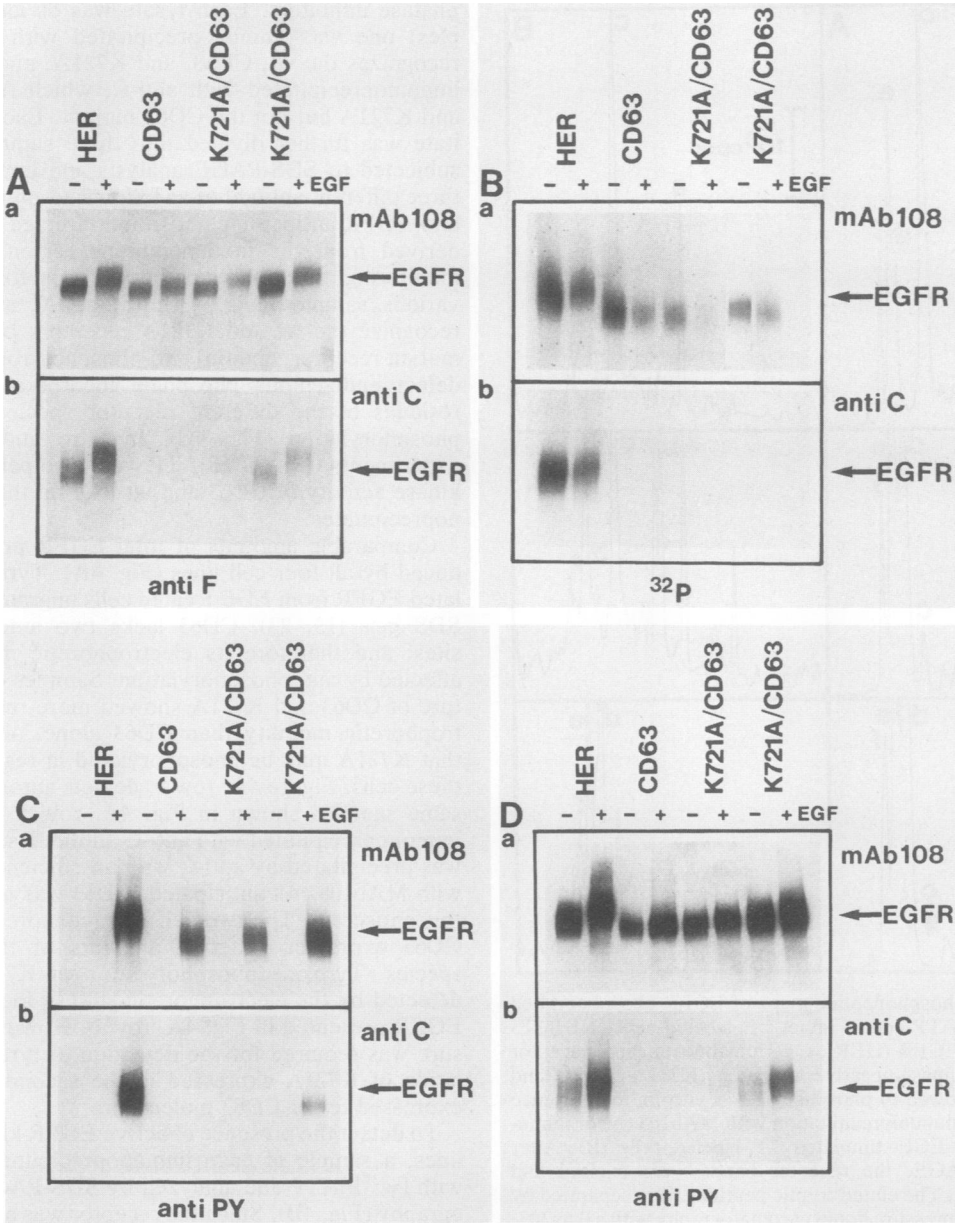

FIG. 4. EGFR autophosphorylation in intact cells: detection by immunoblotting with anti-phosphotyrosine antibodies. NIH 3T3 cells expressing wt EGFR (HER), C-terminally truncated active receptor (CD63), or two different cell lines coexpressing kinase-negative and active receptor (K721A/CD63 cells) were treated overnight with sodium orthovanadate, stimulated in the absence or presence of 500 ng of EGF for 10 min at 37°C, and lysed in the presence of phosphatase inhibitors. EGF receptor was immunoprecipitated with MAb108, which recognize all forms of EGFR (rows a) or with anti-C, which recognizes the wt receptor and K721A mutant (rows b). After separation by SDS-PAGE, the receptors were visualized by immunoblotting. (A) Anti-F used for immunoblotting to reveal the amount of receptor protein present in the different samples. (B) EGFR immunoprecipitates phosphorylated in vitro with [γ-^{32}P]ATP to test for the presence of kinase activity. (C) Anti-phosphotyrosine antiserum used to detect tyrosine phosphorylated proteins. (D) Anti-phosphotyrosine blots of cells treated with phenylarsine oxide (10 μM, 15 min at 37°C) before stimulation with EGF.

tion in intact cells and stimulated the cross-phosphorylation of K721A by CD63 (Fig. 4D). PAO is a dithiol-specific reagent that inhibits receptor internalization (38). Even at 100 mM, PAO did not show any direct effect on kinase activity of purified EGFR, nor did it inhibit tyrosine phosphatase activity of cell lysates (data not shown).

[^{3}H]thymidine incorporation into DNA. We compared the effect of the presence of the kinase-negative EGFR mutant (K721A) on the capacity of active EGFR to stimulate DNA synthesis. We previously demonstrated that EGF-induced mitogenicity is dependent on the number of receptors expressed in each cell line (8). Maximal mitogenic response to saturating amounts of EGF was proportional to receptor number with a plateau at approximately 500,000 receptors per cell (8). We have therefore normalized all the dose-response curves for EGF for maximal mitogenic stimulation after subtraction of basal activity. In this representation, the dose required for half-maximal stimulation depends on the properties of the mutant receptor and not on the level of expression of EGFR (8). For the sake of comparison, we

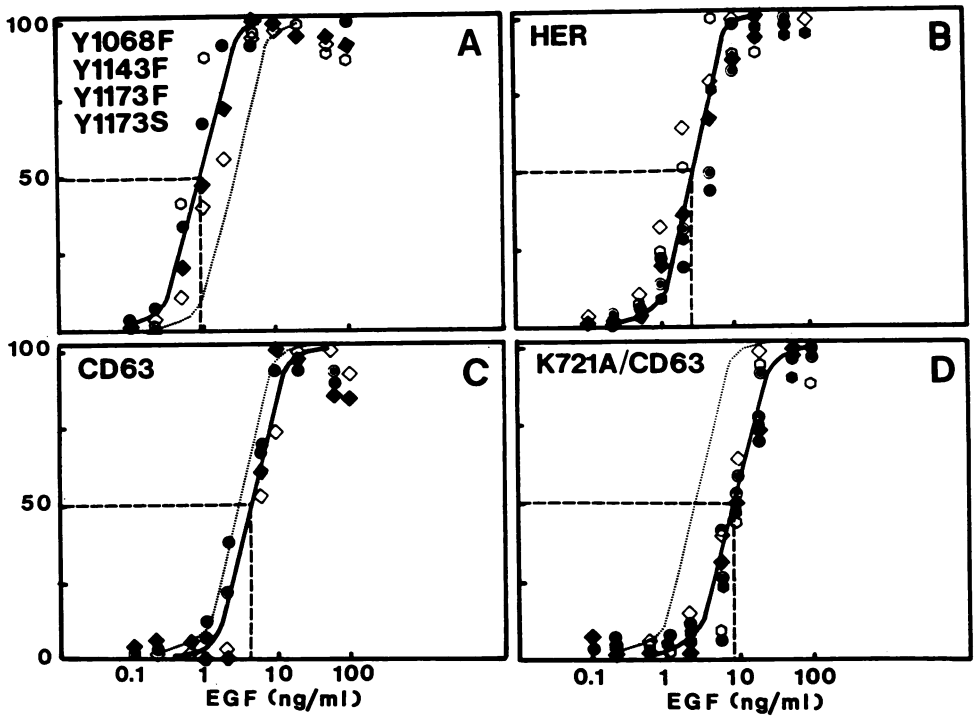

FIG. 5. EGF-stimulated [^{3}H]thymidine incorporation into DNA. NIH 3T3 cells expressing different point mutant EGFR with individually altered autophosphorylation sites (A), wt EGFR (B), or C-terminally truncated active receptor (C) and cells coexpressing the kinase-negative and active receptors (D) were starved to quiescence and then stimulated with different amounts of EGF. At 18 h after the addition of EGF, [^{3}H]thymidine was added to the cells, and 4 h later the amount of radioactivity incorporated into DNA was determined. Dose-response curves of different cell lines were compared by analyzing normalized mitogenic stimulation (8). Numeric results and statistic analyses are presented in Tables 1 and 2.

also included in this analysis previously described cell lines that express mutant EGFR with individually altered tyrosine autophosphorylation sites 1173, 1148, and 1068 (8). According to this analysis, cells expressing wt EGFR (six cell lines) required 3.03 ± 0.9 ng of EGF per ml for half-maximal stimulation of DNA synthesis (Fig. 5B), whereas mutant receptors with individually altered autophosphorylation sites required 0.78 ± 0.27 ng of EGF per ml for half-maximal mitogenic response (Fig. 5A; four cell lines). CD63-expressing cells (five cell lines) were slightly less responsive than cells expressing wt EGFR, requiring 4.45 ± 1.0 ng of EGF per ml (Fig. 5C). The coexpression of K721A caused a decrease in the sensitivity of cells expressing both CD63 and K721A mutant receptors; 10.15 ± 2.66 ng of EGF per ml was required for half-maximal stimulation of DNA synthesis in these cells (seven cell lines) (Fig. 5D). Statistical analysis of the results is presented in Tables 1 and 2. The significance of the dose-response shifts between the various cell lines expressing wt receptor, mutants with altered autophosphorylation sites, the CD63 mutant, and K721A/CD63 was well above 95%.

DISCUSSION

The cytoplasmic portion of EGFR contains a catalytic domain that is endowed with intrinsic protein tyrosine kinase activity (7, 32, 33, 41). The protein tyrosine kinase domain is flanked by two regions that are probably involved in the regulation of receptor function. The upstream juxtamembrane region contains kinase C phosphorylation site(s) (7, 41), whereas the downstream region contains four tyrosine autophosphorylation sites (5, 22). The enzymatic activity of the protein tyrosine kinase is vital for the biological function of the receptor (3, 9, 12, 20, 21, 27, 35). Autophosphorylation is a feature shared by many kinases (1, 15, 25, 29, 30). In some kinases, tyrosine phosphorylation has a regulatory effect on either enzymatic activity (1, 15, 25, 29, 30) or substrate-binding affinity (8, 10). After ligand binding, EGFR is able to form relatively stable oligomers (32, 33). It has been shown that ligand-induced oligomerization plays a role in the process of ligand induced activation of the receptor kinase (32, 33). This introduces a bimolecular component to the overall reaction kinetics, irrespective of the mechanism of the actual autophosphorylation reaction. Therefore, the analysis of reaction kinetics alone cannot give a clear answer as to the mechanism of receptor autophosphorylation (11, 34). Using a different approach, we were able to demonstrate that in vitro receptor autophosphorylation can occur by an intermolecular process (11).

In a living cell, the EGFR is subjected to different conformational constraints as compared with in vitro conditions. In the plasma membrane the freedom of receptor motion is restricted to the two-dimensional array of the lipid bilayer, which also constrains the receptor to a nearly parallel arrangement. We examined whether autophosphorylation in living cells is mediated by an intra- or intermolecular mechanism. To be able to answer this question, we established transfected NIH 3T3 cells that coexpress active EGFR and a kinase-negative receptor mutant. To distinguish between these two molecules, we chose an active EGFR mutant that could be distinguished from the kinase-negative mutant by immunological means and by its pattern of autophosphorylation sites. The CD63 mutant is lacking 63 amino acids from

TABLE 1. Dose of EGF required for half-maximal stimulation of [^{3}H]thymidine incorporation

Description	Cell line	Clone	EGF concn for half-maximal stimulation[a] (ng/ml)
Mutants with altered autophosphorylation sites	NIH Y1068F	8	0.8
	NIH Y1148F	13	0.6
	NIH Y1173F	2:3	1.2
	NIH Y1173S	8	0.5
Wt receptor	NIH HER	2	1.8
	NIH HER	3	2.9
	NIH HER	7	4.3
	NIH HER	9	4.3
	NIH HER	11	2.0
	NIH HER		2.9
CD63	NIH CD63	4	4.6
	NIH CD63	9	3.3
	NIH CD63	12	6.0
	NIH CD63	24	3.9
Cells expressing both K721A and CD63 mutants	K721A/CD63	6	12.5
	K721A/CD63	10	14.0
	K721A/CD63	23	9.5
	K721A/CD63	34	9.5
	K721A/CD63	37	11.8
	K721A/CD63	44	8.4

[a] Mean EGF concentrations ± standard deviations required for half-maximal stimulation were as follows: for mutants with altered autophosphorylation, 0.78 ± 0.27 ng/ml; for the wt receptor, 3.03 ± 0.19 ng/ml; for CD63 cells, 4.45 ± 1.00; for cells expressing K721A and CD63 mutants, 10.15 ± 2.66 ng/ml.

its carboxy terminus and is therefore devoid of two autophosphorylation sites, leading to an altered phosphopeptide map. Whereas antibodies MAb108, anti-F, and RK-2 recognized both wt and truncated receptors, the epitope recognized by anti-C resided in the deleted region. Therefore K721A or wt receptors can be separated from CD63 by selective immunoprecipitation with the anti-C antiserum. Immunoprecipitates from cells labeled with $^{32}P_i$ and stimulated with either PMA or EGF were analyzed by SDS-PAGE (Fig. 1). The phosphoamino acid compositions (Fig. 2) and tryptic phosphopeptide maps (Fig. 3) of the different receptor species were determined. All phosphopeptides seen in the in vitro phosphorylated samples were also detected in maps of EGFR phosphorylated in intact cells (Fig. 3). The phosphopeptide map of CD63 lacked peptides containing Y1173 and Y1148. Mutant receptor K721A phosphorylated by CD63 and then separated by selective immunoprecipitation with anti-C antibodies was phosphorylated on tyrosine and gave a map similar to the phosphopeptide map of the autophosphorylated wt receptor. Although it is impossible to rule out the involvement of additional tyrosine kinases, our data strongly suggest that in intact cells autophosphorylation of EGFR is mediated by an intermolecular mechanism.

TABLE 2. Significance of differences in thymidine incorporation data of wt and EGFR mutants[a]

Cell line	Significance of difference in thymidine incorporation (%)			
	wt	CD63	K721A/CD63	K721A/CD63
Y → F,S	98.6	>99.9	>99.9	>99.9
wt		84.1	99.4	>99.9
CD63			95.4	99.8

[a] The concentration of EGF leading to half-maximal stimulation of [^{3}H] thymidine incorporation into DNA was determined from the dose-response curve of each cell line (Fig. 5). Means and standard deviations for each of the groups were calculated, and the significance of the difference between the results obtained from each pair of groups was estimated from the overlap of the two distribution curves. Y → F,S represents a mutant with altered tyrosine autophosphorylation sites to either phenylalanine or serine. Significance is defined as percentage of data points in each group that lie within error distribution and not overlapping with the error distribution of the second curve.

Although cross-phosphorylation of the kinase-negative receptor by the kinase-active receptor moiety could undoubtedly be seen, quantitation with anti-phosphotyrosine antibodies indicated that the extent of cross-phosphorylation was low considering the proportion of the two receptor species in the transfected cells. Earlier experiments provided an explanation for the low stoichiometry of cross-phosphorylation (9). The kinase-negative mutant has a different endocytic pathway than wt EGFR. Both the wt and the kinase-negative mutant are internalized. However, whereas the normal receptor is delivered to the lysosome and degraded with a half-life of approximately 1 h, the kinase-negative mutant sheds its ligand after endocytosis and recycles to the cell surface within minutes after internalization (9). Chemical covalent cross-linking experiments of EGFR in intact cells revealed the consequence of this difference: wt receptors can be cross-linked at 37°C as efficiently as at low temperatures. Once oligomerized in response to EGF, they remain in close contact during at least part of their endocytotic itinerary. Kinase-negative receptors, in contrast, appear to redisperse rapidly after recycling to the cell surface. When internalization was blocked either by incubation at low temperature or by pretreatment with PAO, the kinase-negative mutant was covalently cross-linked to the same extent as the wt receptors, whereas at 37°C cross-linking of K721A mutants was marginal as compared with that of wt receptors. We have recently shown (Honegger et al., in press) that the two receptor species retain their separate pathways when coexpressed in the same cell. Various experimental approaches indicate that hetero-oligomers of EGFR are formed on the cell surface (18; Honegger et al., in press). However, upon internalization K721A is quickly separated from active receptor and redisperses on the cell surface. Therefore the lifetime of heterodimers composed of K721A and CD63 mutant is greatly reduced as compared with the lifetimes of homodimers of CD63 or homodimers of the wt receptor. Upon inhibition of internalization by low temperature or PAO treatment (38), the concentration of heterodimers on the cell surface actually represents the interactions between wt receptor monomers. Incubation at low temperature or PAO treatment led to a drastic increase in cross-phosphorylation and to covalent cross-linking of K721A and CD63 in the presence of chemical cross-linking agents. The difference in cellular trafficking and surface dynamics between active and kinase-negative mutants indicates that cells expressing K721A are not the best model system for quantitative analysis of intermolecular autophosphorylation in living cells. Therefore, the extent of cross-phosphorylation observed with this model system most likely represents a lower limit to the extent of cross-phosphorylation occurring between wt receptors. Nevertheless, our results clearly show that receptor cross-phosphorylation does occur in living cells. Yet, an intermolecular contribution for receptor autophosphorylation cannot be excluded. However, the virtual identity of the phosphopeptide maps of wt receptor and the cross-phosphorylated kinase-negative EGFR mutant suggests that autophosphorylation is mediated exclusively by an intermolecular process.

Utilizing EGFR mutants lacking individual autophosphorylation sites, we were able to show that the number of intrinsic tyrosine residues competing with cellular substrates for access to the kinase active site has a direct effect on sensitivity of the mitogenic response to EGF stimulation (8, 10). Replacement of an individual tyrosine with phenylalanine residue leads to a two- to threefold decrease in the dose of EGF required for half-maximal stimulation of [^{3}H]thymidine incorporation (8, 10). If receptor cross-phosphorylation is, indeed, responsible for the majority of receptor autophosphorylation in living cells, the introduction of additional autophosphorylation sites could, in principle, increase competitive inhibition and thus increase the dose of EGF required for stimulation of DNA synthesis. Figure 5 and Tables 1 and 2 show that this is indeed the case. Cell lines coexpressing CD63 and K721A require more EGF for half-maximal stimulation than do cell lines expressing the CD63 mutant alone. A similar observation was made with a cell line that expresses the kinase-negative mutant K721A together with CD126, a deletion mutant lacking 126 carboxy-terminus amino acids and four autophosphorylation sites (data not shown). Although CD63 has lost two autophosphorylation sites, the dose required for stimulation of DNA synthesis is slightly higher than that of wt EGFR. Truncation of 63 amino acids not only deletes two autophosphorylation sites but probably also perturbs the structure of the cytoplasmic domain, reducing the activity of the kinase domain for cellular substrates. A similar result was obtained with transfected cells expressing CD126 (data not shown). This mutant receptor is even less sensitive to EGF than CD63, but the coexpression of K721A in cells expressing CD126 further reduces their mitogenic responsiveness toward EGF. Similar results were observed in cells expressing kinase-negative mutant insulin receptors. The presence of kinase-negative mutants suppressed insulin-induced short-term and long-term responses in the transfected cells (2, 6, 24, 31).

How can we explain the effects of K721A coexpression on EGF-induced mitogenesis? We have evidence that the binding of EGF to cells expressing CD63 and K721A leads to the generation of hetero-oligomers of CD63 and K721A, as revealed by covalent cross-linking experiments. The hetero-oligomeric receptors may not be active, thus diminishing the amounts of active homo-oligomers, which in turn will reduce the mitogenic response toward EGF. We have shown that the expression levels of an active receptor molecule, and therefore the concentration of active oligomers, affects the magnitude of the mitogenic response. However, the dose dependence of the mitogenic response is remarkably constant for different cell lines expressing the same EGFR mutant. Therefore, inactivation of wt receptor molecules in inactive heterodimers should lead to a decreased response to saturating EGF concentrations, whereas the half-maximal stimulating dose should remain constant.

Alternatively, autophosphorylation sites on the kinase-negative receptor may serve as a substrate for the active kinase and compete with important cellular substrates in the same way that the intrinsic sites of the active receptor modulate its substrate affinity. Our results with cell lines expressing different mutants with altered autophosphorylation sites indicate that alteration of the apparent substrate affinity of the receptor kinase correlates with a shift in the dose-response curve for mitogenesis toward higher concentrations of EGF (8, 10). The maximal response, however, was not significantly affected, probably because maximal receptor activation compensated for the effects of the mutations. Active receptors in heterodimers would therefore be more strongly inhibited than in homodimers, leading to a dose-response shift that depends on the ratio of the amount of homodimers versus heterodimers. According to this model, cells that coexpress low amounts of K721A should behave like cells that express only wt receptors. With increasing amounts of K721A, the mitogenic dose-response curve should shift until practically all active receptors are present in the form of heterodimers. Under these conditions, further increases in the expression level of K721A would no longer affect the dose-response curve.

An additional explanation is that the kinase-negative K721A mutant may reduce the efficiency of the active kinase by interacting with substrates, thus creating unproductive complexes and protecting key substrates from phosphorylation by active kinase. This consideration is important if substrate concentration is low and therefore the binding of K721A will be able to limit the concentration of the free substrate. In this case, K721A should suppress the maximal response. At excess concentrations, K721A should completely abolish the mitogenic response of the active receptor. Our results seem to favor the second mechanism, although analysis of more cell lines expressing a wider range of active and inactive receptors is required to conclusively exclude other explanations.

It is noteworthy, however, that the effect of K721A coexpression on the biological responsiveness of active EGF receptor is surprisingly large, considering the transient nature of the interaction between active and inactive receptors (Honegger et al., in press). This may indicate that the initial events of signal transduction occur very early after receptor stimulation, perhaps at the level of the plasma membrane, before active and inactive receptors have separated in the course of their different endocytic itineraries (Honegger et al., in press).

In summary, we used site-directed mutagenesis and several independent approaches to demonstrate that EGF induces cross-phosphorylation of EGFR molecules in living cells. Although we cannot completely rule out an intramolecular contribution, it appears that EGFR autophosphorylation both in vitro and in living cells is mediated predominantly by an intermolecular process. The mitogenic response toward EGF is reduced upon coexpression of the K721A mutant in cells expressing active receptor, indicating that the kinase-negative mutant interferes with either receptor activation or signal transduction required for mitogenic stimulation. This implies that the important events of signal transduction occur before K721A and the active receptor are separated by their different endocytic itineraries.

LITERATURE CITED

1. **Cartwright, C. A., W. Eckhardt, S. Simons, and P. L. Kaplan.** 1987. Cell transformation by pp60 c-src mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.
2. **Chou, D. K., T. J. Dull, D. S. Russell, R. Gherzi, D. Lebwohl, A. Ullrich, and O. M. Rosen.** 1987. Human insulin receptors mutated at the ATP binding site lack protein tyrosine kinase activity and fail to mediate post receptor effects of insulin. J. Biol. Chem. **262**:1842–1847.
3. **Chen, S. W., S. C. Lazar, M. Poenie, R. Y. Tsien, G. Gill, and M. G. Rosenfeld.** 1987. Requirement for intrinsic protein tyrosine kinase activity for the immediate and late actions of EGF-receptor. Nature (London) **328**:820–823.
4. **Cochet, C., O. Kashles, E. M. Chambaz, I. Borrello, C. R. King, and J. Schlessinger.** 1988. Demonstration of epidermal growth factor induced receptor dimerization in living cells using a chemical cross-linking agent. J. Biol. Chem. **263**:3290–3295.
5. **Downward, J., P. Parker, and M. D. Waterfield.** 1984. Autophosphorylation sites on the epidermal growth factor receptor.

Nature (London) 311:483–485.
6. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162, 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 45:721–732.
7. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domain. Science 241:48–52.
8. **Honegger, A. M., T. J. Dull, F. Bellot, E. Van Obberghen, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger.** 1988. Biological activities of EGF receptor mutants with individually altered autophosphorylation sites. EMBO J. 7:3045–3052.
9. **Honegger, A. M., T. J. Dull, S. Felder, E. Van Obberghen, F. Bellot, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger** 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell 51:199–209.
10. **Honegger, A. M., T. J. Dull, D. Szapary, A. Komorya, R. Kris, A. Ullrich, and J. Schlessinger.** 1988. Kinetic parameters of the protein tyrosine kinase activity of EGF receptor mutants with individually altered autophosphorylation sites. EMBO J. 7: 3053–3060.
11. **Honegger, A. M., R. M. Kris, A. Ullrich, and J. Schlessinger.** 1988. Evidence that autophosphorylation of solubilized receptors for epidermal growth factor is mediated by intermolecular cross-phosphorylation. Proc. Natl. Acad. Sci. USA 86:925–929.

11a. **Honegger, A. M., A. Schmidt, A. Ullrich, and J. Schlessinger.** 1990. Separate endocytic pathways of kinase defective and active EGF-receptor mutants expressed in the same cells. J. Cell Biol. 110:1541–1548.

12. **Honegger, A. M., D. Szapary, A. Schmidt, R. Lyall, E. Van Obberghen, T. J. Dull, A. Ullrich, and J. Schlessinger.** 1987. A mutant epidermal growth factor receptor with defective protein tyrosine kinase is unable to stimulate proto-oncogene expression and DNA synthesis. Mol. Cell. Biol. 7:4568–4571.
13. **Hunter, T., and J. A. Cooper.** 1981. Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. Cell 24:741–752.
14. **Hunter, T., and J. A. Cooper.** 1985. Protein tyrosine kinases. Annu. Rev. Biochem. 54:897–930.
15. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60 c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell 49:65–73.
16. **Koland, J. G., and R. A. Cerione.** 1988. Growth factor control of epidermal growth factor receptor kinase activity via an intramolecular mechanism. J. Biol. Chem. 263:2230–2237.
17. **Kris, R., I. Lax, M. Gullick, M. D. Waterfield, A. Ullrich, M. Fridkin, and J. Schlessinger.** 1985. Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF-receptor and v-erbB protein. Cell 40:619–625.
18. **Lax, I., F. Bellot, A. M. Honegger, A. Schmidt, A. Ullrich, D. Givol, and J. Schlessinger.** 1990. Domain deletion in the extracellular portion of EGF-receptor reduces ligand binding and impairs cell surface expression. Cell Regul. 1:173–188.
19. **Lax, I., R. Kris, I. Sasson, A. Ullrich, M. J. Hayman, H. Beug, and J. Schlessinger.** 1985. Activation of c-erbB in avian leukosis virus-induced erythroblastosis leads to the expression of a truncated EGF receptor kinase. EMBO J. 4:3179–3182.
20. **Livneh, E., R. Prywes, O. Kashles, N. Reiss, I. Sasson, Y. Mory, A. Ullrich, and J. Schlessinger.** 1986. Reconstitution of human epidermal growth factor receptors in cultured hamster cells. J. Biol. Chem. 261:12490–12497.
21. **Livneh, E., N. Reiss, E. Berent, A. Ullrich, and J. Schlessinger.** 1987. An insertional mutant of epidermal growth factor receptor allows dissection of diverse functions. EMBO J. 6:2669–2676. (Erratum: EMBO J. 6:3202, 1987.)
22. **Margolis, B. L., I. Lax, R. Kris, M. Dombalagian, A. M. Honegger, R. Howk, D. Givol, A. Ullrich, and J. Schlessinger.** 1989. All autophosphorylation sites of epidermal growth factor (EGF) receptor and HER2/neu are located in their carboxy-terminal tails: identification of a novel site in EGF receptor. J. Biol. Chem. 264:10667–10671.
23. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF-receptor signalling. Cell 57:1101–1107.
24. **McClain, D. A., H. Maegawa, J. Lee, T. J. Dull, A. Ullrich, and J. M. Olefksy.** 1987. A mutant insulin receptor with defective tyrosine kinase displays no biologic activity and does not undergo endocytosis. J. Biol. Chem. 262:14663–14671.
25. **Meckling-Hansen, K., R. Nelson, P. Branton, and T. Pawson.** 1987. Enzymatic activation of Fujinami sarcoma virus gag-fps transforming proteins by autophosphorylation at tyrosine. EMBO J. 6:659–666.
26. **Meisenheder, J., P.-G. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein tyrosine kinases both *in vivo* and *in vitro*. Cell 57:1109–1122.
27. **Moolenaar, W. H., A. J. Biermann, B. C. Tilly, I. Verlaan, L. H. Defize, A. M. Honegger, A. Ullrich, and J. Schlessinger.** 1988. A point mutant at the ATP binding site of the EGF receptor abolishes signal transduction. EMBO J. 7:707–710.
28. **Northwood, I. C., and R. J. Davis.** 1989. Activation of the epidermal growth factor receptor tyrosine protein kinase in the absence of receptor oligomerization. J. Biol. Chem. 263:7450–7453.
29. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of pp60 c-src. Cell 49:75–82.
30. **Rosen, O. M., R. Herrera, Y. Olowe, L. M. Petruzzelli, and M. H. Cobb.** 1983. Phosphorylation activates the insulin receptor tyrosine kinase. Proc. Natl. Acad. Sci. USA 80:3237–3240.
31. **Russell, D. S., R. Gherzi, E. L. Johnson, C. K. Chou, and O. M. Rosen.** 1987. The protein tyrosine kinase activity of insulin receptor is necessary for insulin-mediated receptor down-regulation. J. Biol. Chem. 262:11833–11840.
32. **Schlessinger, J.** 1986. Allosteric regulation of the epidermal growth factor receptor kinase. J. Cell Biol. 103:2067–2072.
33. **Schlessinger, J.** 1988. Signal transduction by allosteric receptor oligomerization. Trends Biochem. Sci. 13:443–447.
34. **Schlessinger, J., Y. Schechter, M. C. Willingham, and I. Pastan.** 1978. Direct visualization of binding, aggregation and internalization of insulin and epidermal growth factor on living fibroblastic cells. Proc. Natl. Acad. Sci. USA 75:2659–2663.
35. **Scimeca, J.-C., R. Ballotti, F. Alengrin, A. M. Honegger, A. Ullrich, J. Schlessinger, and E. Van Obberghen.** 1989. Metabolic effects induced by EGF in cells expressing EGF-receptor mutants. J. Biol. Chem. 264:6831–6835.
36. **Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Liberman, J. Schlessinger, J. Downward, J. L. V. Mayes, M. D. Waterfield, N. Wittle, and P. H. Seeburg.** 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature (London) 309: 418–425.
37. **Wahl, M. I., T. O. Daniel, and G. Carpenter.** 1988. Antiphosphotyrosine recovery of phospholipase C activity after EGF treatment of A431 cells. Science 241:968–970.
38. **Walker, F., and A. W. Burgess.** 1988. Transmodulation of the epidermal growth factor receptor in permeabilized 3T3 cells. Biochem. J. 256:109–115.
39. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryonts and leucocytes. Cell 16:777–785.
40. **Yarden, Y., and J. Schlessinger.** 1987. Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. Biochemistry 26:1443–1451.
41. **Yarden, Y., and A. Ullrich.** 1988. Growth factor receptor tyrosine kinases. Annu. Rev. Biochem. 57:443–478.
42. **Zidovetzky, R., Y. Yarden, J. Schlessinger, and T. M. Jovin.** 1986. Microaggregation of hormone-occupied epidermal growth factor receptor on plasma membrane preparations. EMBO J. 5:247–250.