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Evidence that autophosphorylation of solubilized receptors for epidermal growth factor is mediated by intermolecular cross-phosphorylation

(protein-tyrosine kinase/receptor oligomerization)

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ABSTRACT **Structurally distinguishable mutants of human epidermal growth factor receptor (EGFR) were used to investigate the mechanism of EGFR autophosphorylation. Mutant receptors generated by site-directed mutagenesis were expressed in transfected NIH 3T3 cells lacking endogenous receptors. After coincubation of cell lysates in the presence or absence of EGF, receptor immunoprecipitates were incubated with [γ-^{32}P]ATP. A kinase-negative mutant EGFR (K721A), in which Lys-721 in the ATP binding site was replaced by an alanine residue, was shown to be phosphorylated in an EGF-dependent manner by an enzymatically active EGFR deletion mutant lacking two autophosphorylation sites. A mutant EGFR lacking the EGF-binding domain as well as the phosphorylation sites also phosphorylated the kinase-negative mutant. In both cases the kinase-negative mutant K721A was phosphorylated on sites virtually identical to the sites that are autophosphorylated by wild-type recombinant or native human EGFRs. With four different site-specific anti-EGFR antibodies, it was shown that deletion mutants devoid of epitopes recognized by the antibodies were coimmunoprecipitated together with wild-type or mutant receptors recognized by the antibodies. This indicates that EGFR oligomers were preserved during immunoprecipitation. On the basis of these results, we propose that autophosphorylation of solubilized EGFR is mediated by intermolecular cross-phosphorylation, probably facilitated by receptor oligomerization.**

The membrane receptor for epidermal growth factor (EGF) is a 170-kDa glycoprotein with intrinsic protein-tyrosine kinase activity. Stimulation of the receptor molecule by EGF leads to autophosphorylation and the phosphorylation of various cellular substrates (reviewed in refs. 1 and 2). Three tyrosine residues were identified as autophosphorylation sites (Tyr-1068, Tyr-1148, and Tyr-1173; ref. 3), but additional tyrosine residues are probably phosphorylated both *in vitro* and *in vivo*. Autophosphorylation is common to growth factor receptors and cytoplasmic protein-tyrosine kinases (reviewed in refs. 4–8). Various approaches were used to demonstrate that EGF stimulates receptor oligomerization both in living cells (9, 10) and in solubilized or purified preparations (11, 12). Moreover, it was shown that receptor oligomerization plays an allosteric regulatory role in signal transduction from the extracellular ligand-binding domain to the cytoplasmic kinase domain, leading to the stimulation of the kinase activity (2, 11–14). If oligomerization is required for kinase activation, even intramolecular autophosphorylation will follow a nonlinear concentration dependence. Similarly, if the receptor population contains mainly dimeric receptors, a linear concentration dependence will be observed independently of whether autophosphorylation is mediated by an intra- or by an intermolecular process. The activation step cannot be dissociated from the actual autophosphorylation process, and therefore kinetic analysis of autophosphorylation as a function of receptor concentration cannot be used to distinguish between a unimolecular or a bimolecular mechanism (6).

The mechanism of EGF receptor (EGFR) autophosphorylation was investigated utilizing mixed populations of EGFR molecules altered by *in vitro* site-directed mutagenesis. In this report we show that enzymatically active receptor mutants were able to phosphorylate a kinase-negative (15, 16) mutant on the same sites that are autophosphorylated by wild-type (WT) EGFR. These results demonstrate that autophosphorylation of solubilized EGFR can proceed by an intermolecular process, probably facilitated by receptor oligomerization.

MATERIALS AND METHODS

Full details concerning the generation of the various constructs encoding WT or mutant receptors (Fig. 1) were previously described (2, 15–19). NIH mouse 3T3 cells lacking endogenous receptors were transfected with the various constructs (2, 15, 16). Detailed descriptions of transfected cell lines (7, 8, 15, 16) and receptor immunoprecipitation (7, 8), autophosphorylation (7, 8, 15), and phosphopeptide map (7, 8) analyses were reported elsewhere.

Several antisera and monoclonal antibodies were used in this study. RK-2 is a rabbit antiserum generated against a synthetic peptide corresponding to residues 984–996 of the human EGFR (20). Anti-N is a rabbit antiserum generated against a synthetic peptide representing the N-terminal sequence of the human EGFR (residues 1–18). Anti-C is a rabbit antiserum generated against a synthetic peptide from the C terminus of the human EGFR (residues 1176–1186). mAb108 is a monoclonal antibody (IgG2a) that recognizes the extracellular domain of the human EGFR but does not crossreact with murine or chicken EGFR (F. Bellot and J.S., unpublished work).

RESULTS

We have generated transfected NIH 3T3 cell lines that express human EGFR deletion mutants in addition to endogenous murine EGFR. Analysis of the *in vitro* protein-tyrosine kinase activity of EGFR expressed in these cell lines indicated that the phosphorylation of the endogenous murine EGFR was increased in the presence of the human EGFR deletion mutants (data not shown). A similar result was obtained when lysates of cells expressing WT human EGFR but not murine EGFR were mixed with lysates of cells expressing NRCD63, a human EGFR double deletion mutant (Fig. 1; refs. 2 and 18). The data show that phosphorylation of WT receptor was enhanced in a mixture containing

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Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; WT, wild type.

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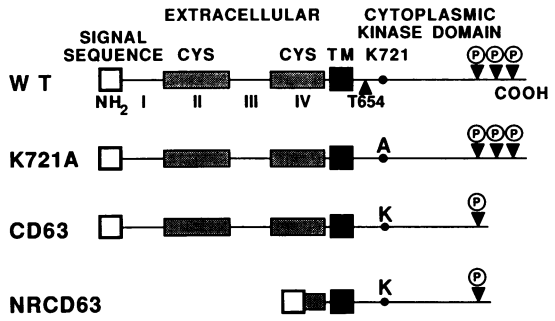

FIG. 1. Schematic representation of human WT EGFR and EGFR mutants. Regions II and IV of the extracellular domain are cysteine-rich. TM, transmembrane domain; K721, Lys-721; A, Ala-721. The three known sites of tyrosine autophosphorylation are indicated by arrowheads above the line. T654 (Thr-654) is phosphorylated by kinase C. K721A is a point mutant (Lys-721 → Ala) devoid of kinase activity (15, 16). CD63 is a truncation mutant devoid of 63 amino acids at the C terminus, lacking two of the three known autophosphorylation sites; this mutant receptor possesses EGF-regulated protein tyrosine kinase activity (formerly called pLSC; ref. 18). NRCD63 is a mutant lacking 520 amino acids from the extracellular ligand-binding domain and devoid of 63 amino acids from the cytoplasmic domain. This mutant EGFR is unable to bind EGF, and therefore its kinase activity is unregulated (2, 18). This mutant is able to phosphorylate its remaining autophosphorylation site(s) and exogenous substrates. The deletions correspond approximately to those found in the v-*erbB* oncogene product.

NRCD63 (Fig. 2 *Left*). This could be the consequence of either direct phosphorylation of WT receptor by the mutant receptor or activation of WT receptor by association with the mutant receptor. The influence of WT receptor on the phosphorylation of the mutant was less pronounced (Fig. 2 *Right*), as could be expected from the lack of two autophosphorylation sites in the truncated receptor (Fig. 1). To determine the mechanism of EGFR phosphorylation, we investigated the phosphorylation of an EGFR mutant devoid of kinase activity by two structurally distinguishable, enzymatically active EGFR mutants.

Fig. 1 gives a schematic representation of the EGFR mutants used for this analysis. WT human EGFR was used as a control for normal receptor autophosphorylation. The phosphopeptide map of WT EGFR was indistinguishable from that of the EGFR of A431 human epidermoid carcinoma cells and was not affected by the type of the anti-EGFR antibodies used for immunoprecipitation (data not shown). K721A is a previously characterized kinase-negative EGFR mutant (15, 16) in which Lys-721 in the ATP binding site of the receptor has been replaced by an alanine residue. In crosslinking experiments, this mutant showed a similar degree of ligand-induced aggregation in intact cells as WT receptor (data not shown) and is therefore fully capable of oligomerization. CD63 and NRCD63, mutants of the EGFR that have kinase activity but lack two of the three identified autophosphorylation sites (2, 18), served as enzymatically active receptors that could be distinguished from the kinase-negative receptor (K721A) by their altered phosphopeptide maps (CD63, NRCD63) and different size (NRCD63). The kinase activity of the NRCD63 mutant cannot be stimulated by EGF, as this mutant receptor lacks most of the ligand-binding domain. The WT and mutant receptors were expressed in an NIH 3T3 cell line lacking endogenous murine EGFR (15, 16). The basic design of the experiments was as follows. Lysates of cells expressing one EGFR mutant were mixed with lysates of cells expressing a different mutant. EGF was added to the reaction mixture and the receptors were immunoprecipitated with site-specific anti-EGFR antibodies recognizing either one or both mutant receptors. The immunoprecipitate was incubated with phosphorylation reaction mixture containing [γ-^{32}P]ATP and Mn^{2+}. The phosphorylated receptors were analyzed by SDS/PAGE followed by autoradiography. Radioactive bands were cut out, digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin and the tryptic phosphopeptides were analyzed by reverse-phase HPLC. Fig. 3*A* shows that the kinase-negative mutant K721A was readily phosphorylated by the NRCD63 mutant, demonstrating intermolecular receptor phosphorylation. An immunoprecipitate of K721A alone or of NRCD63 alone showed no phosphorylated polypeptide of the size of WT receptor, but an immunoprecipitate of a mixture of the two receptor mutants clearly showed a polypeptide of the size of the phosphorylated WT EGFR.

The phosphopeptide map of WT EGFR (Fig. 3*B Top*) revealed the presence of multiple phosphorylation sites. Peaks c, d, and g represent tryptic phosphopeptides containing the previously identified (3) phosphorylation sites at Tyr-1173, Tyr-1068, and Tyr-1148, respectively. The assignment of the phosphorylation sites is based on a comparison with phosphopeptide maps of EGFR mutants in which individual autophosphorylation sites were replaced by phenylalanine residues (7, 8). The additional phosphopeptides observed in this map have not been identified. Mutant receptor NRCD63, which lacks 63 amino acids at the C terminus and therefore lacks the autophosphorylation sites at

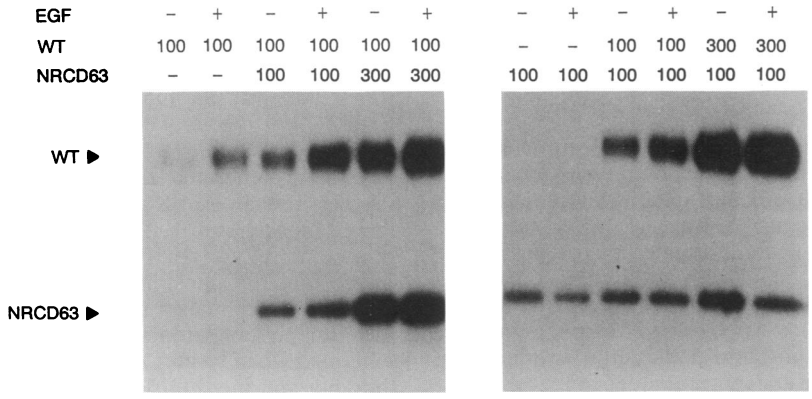

FIG. 2. Phosphorylation of EGFR in lysates containing EGFR mutant NRCD63. (*Left*) A constant amount of WT EGFR lysate (100 μl) was mixed with various amounts (0, 100, or 300 μl) of lysate containing EGFR mutant NRCD63 and lysis buffer to adjust for constant volume. (*Right*) A constant amount of NRCD63-containing lysate (100 μl) was incubated with various amounts (0, 100, or 300 μl) of lysate containing wild-type EGFR. The mixed lysates were incubated in the absence (−) or presence (+) of EGF (1 μg/ml) for 10 min at 30°C and then subjected to immunoprecipitation with anti-EGFR antibodies (RK-2) coupled to protein A-Sepharose beads. The washed immunoprecipitates were incubated with 5 μM ATP, 3 μCi of [γ-^{32}P]ATP (1 μCi = 37 kBq), and 5 mM $MnCl_2$ for 1 min on ice, SDS sample buffer was added, and the phosphorylated receptor was analyzed by SDS/PAGE (5–10% gradient gel) and autoradiography.

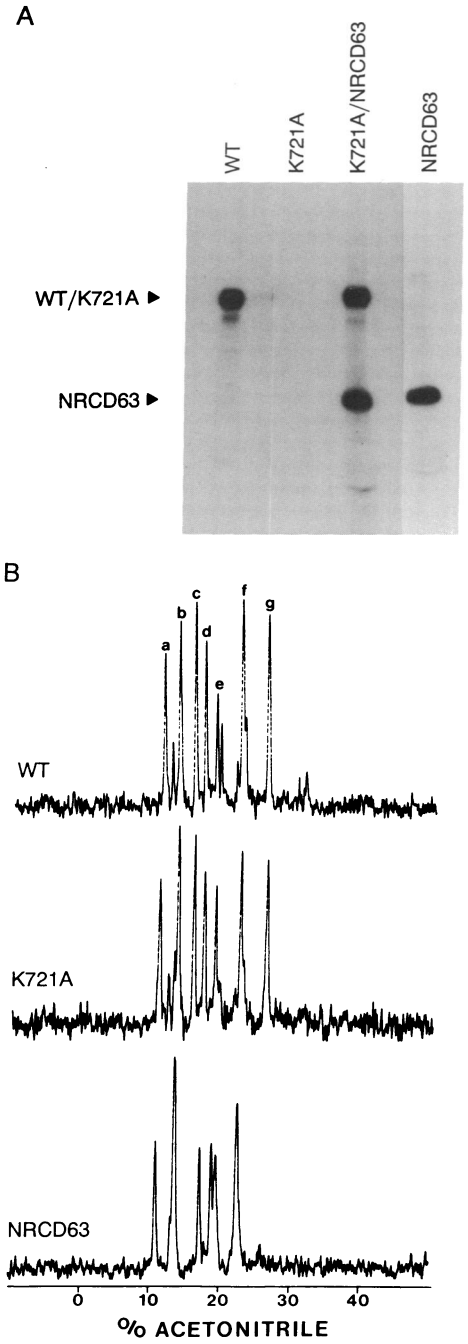

FIG. 3. Phosphorylation of the kinase-negative EGFR mutant K721A by EGFR deletion/truncation mutant NRCD63. (*A*) Autoradiograph of SDS/PAGE of phosphorylated WT EGFR, kinase-negative mutant K721A, mixture of K721A and truncation mutant NRCD63, and NRCD63 alone. (*B*) Tryptic phosphopeptide map of receptor polypeptides isolated from the gel depicted in *A*. Radioactive bands were cut from the dried gel, SDS was eluted with 10% methanol, and the receptor polypeptides were digested in the gel with trypsin in 50 mM ammonium bicarbonate buffer. Tryptic peptides were dried, redissolved in 0.1% trifluoroacetic acid, and analyzed by reverse-phase HPLC on a C_{18} column, using a linear gradient of 0–30% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Radioactive peptides were detected by an on-line liquid scintillation β-counter. (*Top*) Phosphopeptide map of autophosphorylated WT EGFR. Peaks c, d, and g were identified as the tryptic peptides containing the autophosphorylation sites Tyr-1173, Tyr-1068 nd Tyr-1148, respectively (7, 8). Peaks a, b, e, and f represent unidentified peptides. (*Middle*) Phosphopeptide map of kinase-negative mutant K721A (170-kDa band) phosphorylated by mutant NRCD63. (*Bottom*) Phosphopeptide map of autophosphorylated mutant NRCD63.

Tyr-1148 and Tyr-1173, lost peaks c and g from its phosphopeptide map (Fig. 3*B Bottom*). Maps of NRCD63 phosphorylated alone or with K721A were identical. The 170-kDa band representing K721A phosphorylated by NRCD63 (Fig. 3*B Middle*) showed a phosphopeptide map strikingly similar to that of "autophosphorylated" WT EGFR; the only visible difference in the phosphopeptide maps is an occasional splitting of peak e into a doublet in both the WT and NRCD63 receptors. Peak e is also occasionally split in the phosphorylated K721A receptor mutant, and therefore this difference is probably insignificant.

EGF Stimulates Receptor Cross-Phosphorylation. Because the NRCD63 EGFR mutant lacks most of the ligand-binding domain, it does not bind EGF and its kinase activity cannot be regulated by the growth factor. To investigate the influence of EGF activation on receptor cross-phosphorylation, mixtures of K721A lysates with mutant CD63 lysates were used. The CD63 EGFR mutant binds ligand normally. EGF is able to stimulate the kinase activity of this receptor mutant and to stimulate DNA synthesis of cells expressing it (18). The difference between the sizes of K721A and CD63 is not sufficient to allow the separation of the two mutants by SDS/PAGE. Fig. 4*A* shows that increasing the proportion of K721A in mixtures containing equal amounts of CD63 caused increased phosphorylation of the 170-kDa receptor band. Moreover, stimulation of receptor phosphorylation by EGF was independent of the K721A/CD63 ratio. Phosphopeptide analyses (Fig. 4*B*) showed that peaks c and g, absent from the CD63 profile, are found as phosphorylated products in K721A/CD63 mixtures, and their intensity is increased upon stimulation with EGF. This indicates that the enzymatically active CD63 EGFR mutant is able to phosphorylate, in an EGF-dependent manner, the kinase-negative K721A EGFR mutant. Moreover, the phosphopeptide map of the cross-phosphorylated receptor is indistinguishable from the phosphopeptide map of the autophosphorylated WT. It contains all the peaks of the autophosphorylated CD63 receptor and the additional peaks contributed by the cross-phosphorylated K721A mutant containing residues Tyr-1148 and Tyr-1173.

Evidence for Coimmunoprecipitation of Mutant Receptors. Antibodies against the C-terminal peptide of the EGFR (Anti-C) do not recognize the CD63 mutant because this determinant was deleted from this mutant receptor. Nevertheless, the Anti-C antibodies were able to coimmunoprecipitate the CD63 mutant in mixtures containing either WT or K721A receptor as efficiently as antipeptide antibody RK-2, which recognizes an antigenic determinant common to WT and K721A and CD63 mutants. Similarly, monoclonal antibodies against the human EGFR (mAb108) do not recognize the murine EGFR but are able to coimmunoprecipitate the murine receptor in a mixture containing the kinase-negative K721A EGFR mutant (Fig. 5). This is indicated by the appearance of kinase activity in the immunoprecipitates of mixtures of K721A and murine EGFR. A similar conclusion was reached in a separate study in which similar experiments were done with a different set of deletion mutants of the human EGFR and EGFR from other species (I. Lax, and J.S., unpublished data). It appears that the association between EGFR molecules is sufficiently strong to withstand not only the immunoprecipitation itself but also the multiple washing steps involved in purifying the immunoprecipitates. We interpret these results as an indication that EGFR molecules are oligomerized and that receptor oligomerization is maintained between different EGFR mutants or between EGFRs of different species.

DISCUSSION

The capacity to catalyze autophosphorylation is common to many protein kinases, including growth factor receptors with intrinsic protein-tyrosine kinase activity (5). The binding of EGF to the extracellular domain leads to the activation of the

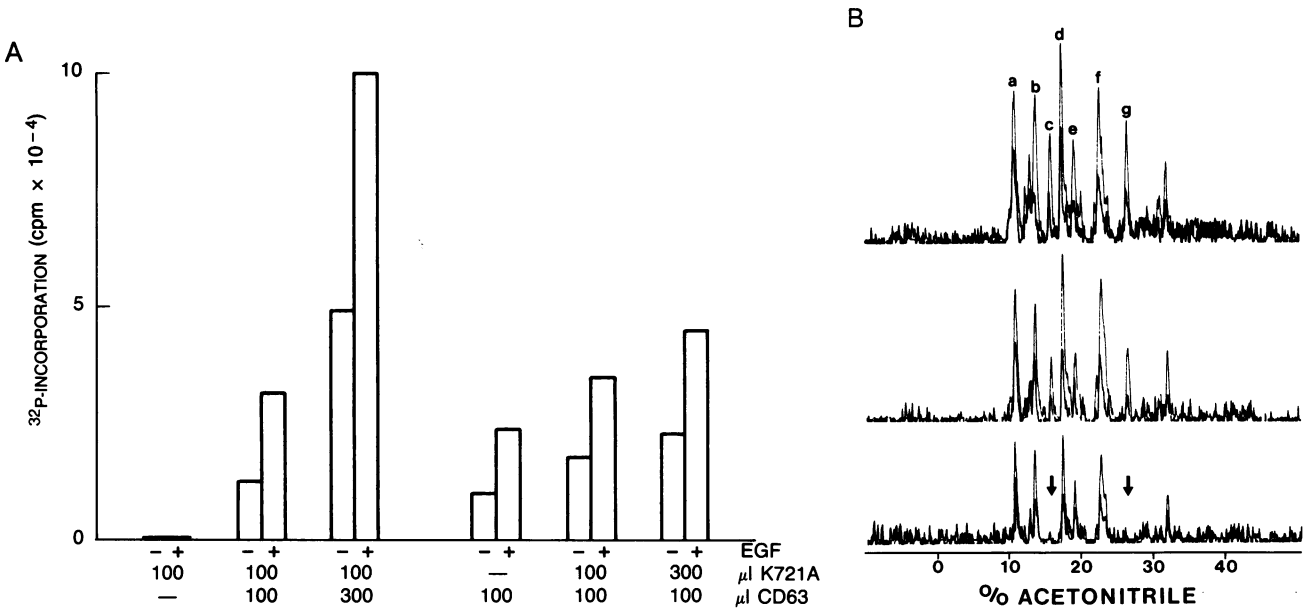

FIG. 4. EGF-induced phosphorylation of the kinase-negative mutant K721A by mutant CD63. A constant amount of K721A lysate (100 μl) was mixed with various amounts of CD63 lysate, or various amounts of K721A lysate mixed with a constant amount of CD63 lysate. The mixtures were incubated with (+) or without (−) EGF (1 μg/ml) and then subjected to immunoprecipitation with antiserum RK-2 and phosphorylation as described for Fig. 1. After SDS/PAGE, the gel was autoradiographed. Radioactive bands were cut out, quantitated by counting Cerenkov radiation, and digested with trypsin. Phosphopeptides were analyzed by HPLC as described in the legend to Fig. 3*B*. (*A*) Histogram depicting the amount of radioactivity in each of the bands in the gel. (*B*) Phosphopeptide maps of the phosphorylated receptor. Thick lines trace the maps obtained from samples phosphorylated without prior activation with EGF. Overlaid profiles in thin lines show maps of samples phosphorylated under identical conditions after activation with EGF. Arrows indicate the position of phosphopeptides containing Tyr-1173 and Tyr-1148 (peaks c and g), which are missing from mutant EGFR CD63 due to the C-terminal truncation. (*Top*) CD63 (100 μl) plus K721A (300 μl). (*Middle*) CD63 (100 μl) plus K721A (100 μl). (*Bottom*) CD63 (100 μl).

cytoplasmic kinase domain, inducing rapid autophosphorylation of the receptor. In addition, EGFR is able to undergo rapid ligand-induced oligomerization (2, 11, 12). It was proposed that binding of EGF to the extracellular domain stabilizes an oligomeric state that facilitates the activation of the cytoplasmic kinase function by receptor–receptor interactions (2, 11, 12). Two distinct mechanisms have been proposed to explain autophosphorylation as a consequence of receptor oligomerization and kinase activation. According to one model, receptor autophosphorylation follows an intramolecular process in which the catalytic domain of EGFR phosphorylates directly the phosphorylation sites of the same molecule (6). Alternatively, autophosphorylation may follow an intermolecular process in which the catalytic domain of one receptor phosphorylates the phosphorylation sites of a neighboring receptor molecule. A conventional approach to distinguish between these two mechanisms is to determine the concentration dependence of receptor autophosphorylation. As already mentioned, such experiments are difficult to interpret (6) because of the tendency of EGFR to undergo oligomerization and because of the role played by receptor oligomerization in the activation of the kinase (11, 12). Either mechanism can lead to apparent unimolecular or apparent higher-order kinetics, depending on whether the initial sample contains predominantly monomers or oligomers.

To avoid these complications, the mechanism of autophosphorylation was explored by using a mixed population of EGFR mutants. The kinase-negative mutant K721A (15, 16) served as a substrate for different structurally distinguishable enzymatically active EGFR mutants. The conclusions drawn

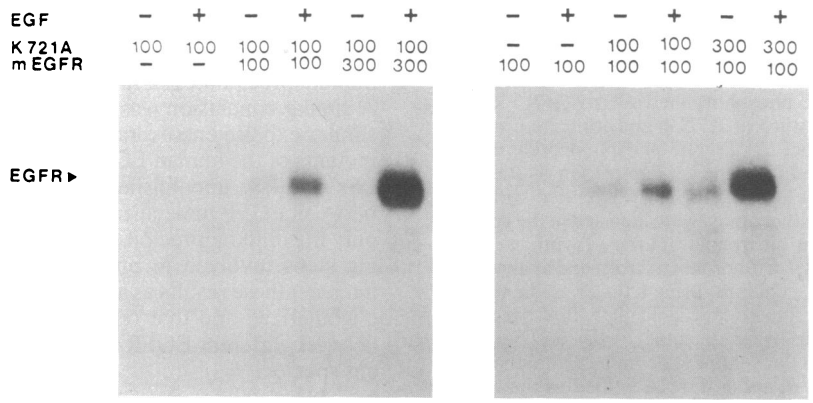

FIG. 5. Coimmunoprecipitation of murine EGFR (mEGFR) with kinase-negative human EGFR (K721A) by human-specific anti-EGFR antibodies. Lysates of an NIH 3T3 cell line expressing endogenous mEGFR were mixed with lysates of NIH 3T3 cells expressing human EGFR mutant K721A. After incubation with or without EGF, receptors were immunoprecipitated with mAb108, which recognizes the human EGFR but not the murine receptor. The presence of kinase activity in immunoprecipitates from mixed lysates demonstrates the coimmunoprecipitation of the enzymatically active mEGFR with the kinase-negative EGFR mutant K721A.

from this analysis are as follows. (*i*) EGFR mutant CD63 phosphorylated, in an EGF-dependent manner, the kinase-negative K721A mutant on sites virtually identical to the sites autophosphorylated by WT or native human EGFR. (*ii*) The EGF-independent mutant NRDC63 phosphorylated the K721A mutant on sites virtually identical to the sites autophosphorylated by WT or native human EGFR. (*iii*) Various site-specific anti-EGFR antibodies coimmunoprecipitated deletion mutants, lacking the epitopes recognized by the antibodies, together with WT or mutant receptors.

Comparison of phosphopeptide maps indicated that in the kinase-negative mutant K721A phosphorylated by NRCD63, the same peptides were phosphorylated as in autophosphorylated WT EGFR. Moreover, even the relative peak heights of the phosphopeptide maps were similar (Fig. 3*B*). Therefore, it was concluded that an EGFR molecule with inactive kinase can be phosphorylated by the active kinase of a second EGFR molecule, and that this phosphorylation is indistinguishable from the autophosphorylation of WT receptor. Similar results were obtained with four different antibodies, indicating that the cross-phosphorylation of the kinase-negative mutant by the enzymatically active receptors is independent of the epitopes recognized by the antibodies used in the standard phosphorylation experiments. Moreover, by using site-specific antibodies we confirmed an earlier result (I. Lax and J.S., unpublished results) indicating that WT receptor and various mutant receptors undergo coimmunoprecipitation even though the antibody used recognizes epitopes found in only one of the mutant receptors. This result further confirms the intrinsic ability of EGFR to oligomerize. It shows that the interactions that stabilize oligomerization are sufficiently strong to allow coimmunoprecipitation of the kinase-positive mutant with the kinase-negative mutant or other receptor mutants.

We emphasize that our results do not exclude an intramolecular contribution for EGFR autophosphorylation. Moreover, it has not yet been shown whether autophosphorylation in living cells occurs by the same mechanism as in the solubilized EGFR. It is clear, however, that upon solubilization, an enzymatically active EGFR mutant was able to phosphorylate the kinase-negative EGFR mutant. The virtual identity of the phosphopeptide maps of WT receptor and of kinase-negative receptor phosphorylated by cross-phosphorylation suggests that autophosphorylation is mediated by an intermolecular process. Although a contribution by an intramolecular mechanism cannot be ruled out, it is unlikely that two different mechanisms responsible for phosphorylation of WT and kinase-negative receptor should yield such similar results. The results demonstrating coimmunoprecipitation of the various mutant EGFRs indicate that EGFR is present in an oligomeric form. By various approaches it was previously shown that EGF stabilizes the oligomeric state of its receptors (2, 11–14, 21), a process that plays a role in the activation step. We propose, therefore, that the intrinsic ability of EGFR to oligomerize, and to do so in response to ligand stimulation, is sufficient to enable receptor molecules to phosphorylate one another. According to this view, both receptor activation and receptor autophosphorylation are facilitated by the same mechanism; namely, by the intrinsic ability of the EGFR to oligomerize.

The effect of autophosphorylation on the kinase activity is small; it can be explained by the removal of intrinsic substrate sites, which compete with external substrates for the active site of the kinase moiety. Consequently, the apparent K_m of external substrates decreases as the internal sites are phosphorylated (7, 8). This leads to an increase in sensitivity to EGF and increased basal DNA synthesis of cells expressing these mutant receptors (7, 8). Whether receptor autophosphorylation has additional functions in the context of a living cell is not clear. Experiments using both *in vitro* systems and cell lines that coexpress two different EGFR mutants will allow analyses of the interactions leading to receptor oligomerization and activation and to phosphorylation of the receptor and other physiologically important substrates.

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